CHAPTER 5

GENE-BASED THERAPY

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Advances in molecular and cellular biology have described the proteins that mediate many disease processes, while DNA technology provides ready access to the genes that control these events. The size, complexity, and cellular inaccessibility of these proteins make their delivery or modification by conventional pharmacological means impossible. Gene therapy overcomes these barriers by the selective introduction of recombinant DNA into tissues so that the biologically active proteins can be synthesized within the cells whose function is to be altered. As such, delivery of recombinant DNA has become a central issue in all gene therapy strategies. A variety of DNA delivery systems have been developed based on viral life cycle pathways, liposome encapsulation, direct injection, and complexation with carrier proteins. Although originally envisioned as a treatment for inherited single-gene defects, gene therapy has found applications in acquired illnesses such as cancer, cardiovascular and infectious diseases. This chapter provides an introduction to the therapeutic issues and current strategies being explored to apply gene therapy to this wide range of diseases.

SCOPE OF GENE THERAPY

Therapeutic gene transfer is not a new concept (Wolff and Lederberg, 1994). More than two decades before the first gene transfer took place in a clinical setting, Edward Tatum speculated: "We can even be somewhat optimistic on the long-range possibility of therapy by the isolation or design, synthesis, and introduction of new genes into defective cells of particular organs" (Tatum, 1966). The treatment of human disease by gene transfer originally was envisioned as a means to treat diseases arising from single-gene defects. Inherited diseases encompass a wide range of disorders wherein a defective gene leads to the failure to synthesize a particular protein or to the synthesis of an abnormal protein. In either event, the absence of the normal protein can lead to a variety of clinical manifestations depending on the structural or enzymatic role that protein normally plays in the cell. Such conditions range from mild disorders that require no treatment (*e.g.*, color blindness) to life-threatening diseases (*e.g.*, hemophilia, cystic fibrosis). These diverse diseases are, in general, inadequately treated by conventional pharmacological means. Therapy based on the replacement of the missing or defective protein (such as factor VIII for hemophilia, transfusions for sickle cell disease, and adenosine deaminase for severe combined immunodeficiency syndrome) is available for only a few of these disorders. Furthermore, these therapies are only partially effective in ameliorating the manifestations of the disease and are accompanied by significant complications. For most genetic diseases, providing the missing protein in a therapeutic fashion is not feasible due to the complex and fragile nature of the protein and the need to deliver the protein to a specific subcellular location (*i.e.*, cell surface expression, lysosomal localization, *etc.*). Transplantation of the major affected organ has been done in some instances (*e.g.*, bone marrow transplantation for sickle cell disease, or liver transplantation for hyperlipidemias), but this also has severe limitations of organ availability and adverse consequences arising from the immune suppression required to prevent rejection of an allogeneic tissue.

Providing a normal copy of the defective gene to affected tissues would circumvent the problem of delivering complex proteins, as the protein could be synthesized within the cells using the normal cellular pathways. Although the defective gene is present in all cells of an individual with an inherited disorder, only a few tissues or organs actually express the gene and therefore are affected. Defects in genes that function in all cells of the body (so-called housekeeping genes) usually result in such severe abnormalities that embryonic development cannot occur. The limited number of tissues affected by most inherited disorders greatly simplifies the requirements for effective gene therapy, since a functional copy of the gene need be

provided only to those tissues that actually require it. The goal of gene therapy, therefore, is to genetically correct the defect in only part of the body. Since this type of therapy is designed not to alter the genetic structure of reproductive organs, it does not prevent the genetic disorder from being passed on to subsequent generations. It is envisioned, however, as a powerful tool to ameliorate or reverse the metabolic consequences in the treated individual. Targeting of the therapeutic gene to a specialized tissue is an area of tremendous interest in all applications of gene therapy. Furthermore, if the gene transfer can be targeted to the major affected organs, then side effects arising from ectopic gene expression in nontarget cells might be avoided. As with other pharmaceutical agents, cell-specific targeting has the advantage of decreasing the effective volume of distribution and the amount of gene transfer agent needed. Such cell-specific delivery systems are not yet available for either drugs or genetic material, but it can be reasonably expected that the explosion of interest in gene therapy will result in new methods that are applicable to the delivery of DNA and conventional pharmaceutics alike. DNA delivery systems are being developed using a variety of chemical, physical, and biologic agents.

The earliest human gene transfer experiments began in 1989 with lymphocyte marking studies. While offering no therapeutic benefit, these initial studies showed that gene transfer could be safely carried out and provided insight into many of the technical difficulties of human gene transfer (Rosenberg *et al.*, 1990). Lymphocytes were likely targets for initial gene therapy attempts because they can be isolated easily and manipulated *ex vivo*. Thus, tissue targeting can be effected by physical removal and manipulation of the recipient cells, rather than by design of the gene delivery system, which has so far proved difficult. Lymphocytes were also attractive because they are the cellular locus of several inherited and acquired disorders (*e.g.*, severe combined immunodeficiency, HIV infection, graft versus host disease, and a variety of malignancies). Furthermore, in addition to being readily isolated, lymphocytes may be expected to be long-lived on return to the recipient and therefore can potentially provide lasting benefits in chronic disorders. Thus, lymphocyte gene transfer provides an important model for gene therapy, and continues to be developed for many disorders. In September 1990, the first human gene therapy trial with therapeutic potential began. The *ex vivo* gene transfer of adenosine deaminase (ADA) gene into the lymphocytes of a child with what is normally a lethal deficiency of this enzyme was carried out at the National Institutes of Health (Anderson *et al.*, 1990). The results of this trial, which are yet to be published in detail, were encouraging and have spawned the development of many new gene therapy trials.

The majority of gene therapy trials under way are for the treatment of acquired disorders such as AIDS, malignancies, and cardiovascular disease, rather than diseases arising from single gene defects (Table 5–1). The application of gene therapy to acquired disorders has proceeded faster than applications for single-gene defects for several reasons. Principle among these reasons is that the long-term gene expression (months to years) that is likely necessary to treat genetic diseases has been difficult to achieve. The availability of a large pool of candidate patients with severe and immediately life-threatening acquired disorders (most notably cancer and AIDS) provides a clinical setting to develop new strategies for DNA delivery that may be applied later to inherited disorders. In contrast to the inherited diseases where a genetic defect has been well characterized, in most applications of gene therapy to acquired illnesses, the molecular basis of the disease is less well understood. Rather than correct a known underlying defect, the approach has been to add new molecular functions that are capable of altering the course of the disease, or to block an existing function, rather than correct an underlying deficiency.

General Considerations in Gene Therapy

Inherited Disorders. The insertion of a new gene that ultimately corrects a deficiency requires that the new gene product is present in sufficient amounts to achieve a therapeutic effect. The level of protein function necessary to achieve complementation of the defect varies widely among genetic diseases. Often this can be estimated from clinical observations comparing the severity of the disease with the extent of deficiency. This is seen in the hemophilias, where the extent of bleeding complications is roughly proportional to the extent of the deficiency. Such estimates are not possible in other disorders such as cystic fibrosis, where the amount of cystic fibrosis transport regulator (CFTR) gene expression, in the airway and in other epithelial cells, necessary to achieve therapeutic benefit is not known. Here, the severity of the illness correlates with the type of genetic defect, rather than with the level of protein expression. These issues become more complex in diseases where gene expression must be carried out in a highly regulated fashion. One such example is the thalassemias, which arise from defects in the synthesis of either the α or β chain of hemoglobin. Excessive production of either subunit chain by an unregulated therapeutic gene transfer could be as harmful as the disease itself.

Acquired Disorders. Mechanistically, gene therapy for acquired disorders is potentially more flexible, in terms of the inserted DNA, than gene therapy for inherited disorders. In inherited disorders, a single defective gene that causes the disorder typically is the subject of intervention. By contrast, in acquired diseases, either a defective gene

Table 5–1

Therapeutic Gene Therapy Trials Approved by the Recombinant DNA Advisory Committee of the National Institutes of Health.*

PROTOCOL TITLE	PRINCIPAL INVESTIGATOR	DATE OF APPROVAL
Gene Therapy of Patients with Advanced Cancer Using Tumor Infiltration Lymphocytes Transduced with the Gene Coding for Tumor Necrosis Factor.	S.A. Rosenberg	7/31/90
Immunization of Cancer Patients Using Autologous Cancer Cells Modified by Insertion of the Gene for Tumor Necrosis Factor (TNF)	S.A. Rosenberg	10/7/91
Immunization of Cancer Patients Using Autologous Cancer Cells Modified by Insertion of the Gene for Interleukin-2 (IL-2).	S.A. Rosenberg	10/7/91
Ex vivo Gene Therapy of Familial Hypercholesterolemia.	J.M. Wilson	10/8/91
Treatment of Severe Combined Immune Deficiency (SCID) Due to Adenosine Deaminase (ADA) Deficiency with Autologous Lymphocytes Transduced with the Human ADA Gene: An Experimental Study	R.M. Blaese	2/10/92
Immunotherapy of Malignancy by *in vivo* Gene Transfer into Tumors	G.J. Nabel	2/10/92
Gene Transfer for the Treatment of Cancer.	S.M. Freeman	2/10/92
Gene Therapy for the Treatment of Recurrent Glioblastoma Multiforme with *in vivo* Tumor Transduction with the Herpes Simplex–Thymidine Kinase Gene/Ganciclovir System.	K.W. Culver	3/1/92
A Phase I Study, in Cystic Fibrosis Patients, of the Safety, Toxicity, and Biological Efficacy of a Single Administration of a Replication Deficient, Recombinant Adenovirus Carrying the cDNA of the Normal Human Cystic Fibrosis Transmembrane Conductance Regulator Gene in the Lung.	R.G. Crystal	5/17/92
Phase I Study of Cytokine-Gene Modified Autologous Neuroblastoma Cells for Treatment of Relapsed/ Refractory Neuroblastoma.	M.K. Brenner	6/1/92
Gene Therapy for the Treatment of Brain Tumors Using Intra-Tumoral Transduction with the Thymidine Kinase Gene and Intravenous Ganciclovir	E. Oldfield	6/1/92
Immunization with HLA-A2 Matched Allogeneic Melanoma Cells that Secrete Interleukin-2 in Patients with Metastatic Melanoma.	B. Gansbacher	6/2/92
Immunization with Interleukin-2 Secreting Allogeneic HLA-A2 Matched Renal Cell Carcinoma Cells in Patients with Advanced Renal Cell Carcinoma.	B. Gansbacher	6/2/92
Clinical Protocol for Modification of Oncogene and Tumor Suppressor Gene Expression in Non-Small Cell Lung Cancer (NSCLC).	J.A. Roth	9/15/92
Gene Therapy of Cancer: A Pilot Study of IL-4 Gene Modified Antitumor Vaccines.	M.T. Lotze	9/15/92
Gene Therapy of Cystic Fibrosis Lung Diseases Using E1 Deleted Adenoviruses: A Phase I Trial.	J.M. Wilson	12/3/92
Cystic Fibrosis Gene Therapy Using an Adenovirus Vector: *In vivo* Safety and Efficacy in Nasal Epithelium.	M.J. Welsh	12/4/92
Phase I Study of Non-Replicating Autologous Tumor Cell Injections Using Cells Prepared With or Without Granulocyte-Macrophage Colony Stimulating Factor Gene Transduction in Patients with Metastatic Renal Cell Carcinoma.	J. Simons	3/1/93
Administration of Neomycin Resistance Gene Marked EBV Specific Cytotoxic T Lymphocytes to Recipients of Mismatched-Related or Phenotypically Similar Unrelated Donor Marrow Grafts.	H.E. Heslop	3/2/93
A Phase I Study of Gene Therapy of Cystic Fibrosis Utilizing a Replication Deficient Recombinant Adenovirus Vector to Deliver the Human Cystic Fibrosis Transmembrane Conductance Regulator cDNA to the Airways.	R.W. Wilmott	3/2/93
Gene Therapy for Cystic Fibrosis Using E1 Deleted Adenovirus: A Phase I Trial in the Nasal Cavity.	R.C. Boucher	3/2/93
A Phase I Trial of Human Gamma Interferon-Transduced Autologous Tumor Cells in Patients With Disseminated Malignant Melanoma.	H.F. Seigler	6/7/93
Use of Safety-Modified Retroviruses to Introduce Chemotherapy Resistance Sequences into Normal Hematopoietic Cells for Chemoprotection During the Therapy of Ovarian Cancer: A Pilot Trial.	A.B. Deisseroth	6/7/93
Immunotherapy for Cancer by Direct Gene Transfer into Tumors	G.J. Nabel	6/7/93
Gene Therapy for Gaucher Disease: *Ex vivo* Gene Transfer and Autologous Transplantation of CD34+ Cells.	J.A. Barranger	6/7/93
Retroviral Mediated Transfer of the cDNA for Human Glucocerebrosidase into Hematopoietic Stem Cells of Patients with Gaucher Disease.	S. Karlsson	6/7/93
A Preliminary Study to Evaluate the Safety and Biologic Effects of Murine Retroviral Vector Encoding HIV-1 Genes [HIV-IT(V)] in Asymptomatic Subjects Infected with HIV-1.	J.E. Galpin	6/7/93

*The protocols listed were approved through August, 1994. Detailed protocols for these clinical trials are published in the monthly journal *Human Gene Therapy*.

(Continues)

Table 5–1
Therapeutic Gene Therapy Trials Approved by the Recombinant DNA Advisory Committee of the National Institutes of Health.[a] (*Continued*)

PROTOCOL TITLE	PRINCIPAL INVESTIGATOR	DATE OF APPROVAL
A Molecular Genetic Intervention for AIDS—Effects of a Transdominant Negative Form of Rev.	G.J. Nabel	6/7/93
Gene Therapy for the Treatment of Recurrent Pediatric Malignant Astrocytomas with *in vivo* Tumor Transduction with the Herpes Simplex–Thymidine Kinase Gene.	C. Raffel	6/8/93
Human MDR Gene Transfer in Patients with Advanced Cancer.	C. Hesdorffer	6/8/93
Gene Therapy for Human Brain Tumors Using Episome-Based Antisense cDNA Transcription of Insulin-Like Growth Factor I.	J. Han	6/8/93
Immunization of Malignant Melanoma Patients with Interleukin 2–Secreting Melanoma Cells Expressing Defined Allogeneic Histocompatibility Antigens.	T.K. Das Gupta	9/10/93
Retroviral Mediated Transfer of the Human Multi-Drug Resistance Gene (MDR-1) into Hematopoietic Stem Cells During Autologous Transplantation after Intensive Chemotherapy for Breast Cancer.	J. O'Shaughnessy	9/9/93
Gene Therapy for Recurrent Pediatric Brain Tumors.	L.B. Kun	9/9/93
A Phase I Clinical Trial to Evaluate the Safety and Effects in HIV-1 Infected Humans of Autologous Lymphocytes Transduced with a Ribozyme that Cleaves HIV-1 RNA.	F. Wong-Staal	9/10/93
Genetically Engineered Autologous Tumor Vaccines Producing Interleukin-2 for the Treatment of Metastatic Melanoma.	J.S. Economou	9/10/93
Intrathecal Gene Therapy for the Treatment of Leptomeningeal Carcinomatosis.	B.H. Oldfield	12/2/93
Injection of Colon Carcinoma Patients with Autologous Irradiated Tumor Cells and Fibroblasts Genetically Modified to Secrete Interleukin-2.	R.E. Sobol	12/2/93
Retrovirus-Mediated Transfer of the cDNA for Human Glucocerebrosidase into Peripheral Blood Repopulating Cells of Patients with Gaucher's Disease.	F. Schuening	12/2/93
An Open Label, Phase I/II Clinical Trial to Evaluate the Safety and Biological Activity of HIV-IT (V) (HIV-1 HBenv/Retroviral Vector) in HIV-1 Infected Subjects.	R. Haubrich	12/3/93
A Phase I Trial of B7-Transfected Lethally Irradiated Allogeneic Melanoma Cell Lines to Induce Cell Mediated Immunity Against Tumor-Associated Antigens Presented by HLA-A1 in Patients with Stage IV Melanoma.	M. Sznol	12/3/93
Phase I Study of Immunotherapy of Advanced Colorectal Carcinoma by Direct Gene Transfer into Hepatic Metastases.	J. Rubin	12/3/93
Adoptive Immunotherapy of Melanoma with Activated Lymph Node Cells Primed *in vivo* with Autologous Tumor Cells Transduced with the IL-4 Gene.	A.E. Chang	12/3/93
Gene Therapy for Cystic Fibrosis Using Cationic Liposome Mediated Gene Transfer: A Phase I Trial of Safety and Efficacy in the Nasal Airway.	E.J. Sorscher	12/3/93
Adenovirus-Mediated Gene Transfer of CFTR to the Nasal Epithelium and Maxillary Sinus of Patients with Cystic Fibrosis.	M.J. Welsh	12/3/93
A Phase I Study of Immunization with Gamma Interferon Transduced Neuroblastoma Cells.	J. Rosenblatt	3/3/94
A Phase I/II Pilot Study of the Safety of the Adoptive Transfer of Syngeneic Gene-Modified Cytotoxic T-Lymphocytes in HIV-Infected Identical Twins.	R. Walker	3/3/94
Expression of an Exogenously Administered Human Alpha-1-Antitrypsin Gene in the Respiratory Tract of Humans.	K. Brigham	3/3/94
Phase I Study of Immunotherapy for Metastatic Renal Cell Carcinoma by Direct Gene Transfer into Metastatic Lesions.	N. Vogelzang	3/4/94
Phase I Study of Immunotherapy of Malignant Melanoma by Direct Gene Transfer.	E. Heath	3/4/94
Phase I Trial of a Polynucleotide Augmented Anti-Tumor Immunization of Human Carcinoembryonic Antigen in Patients with Metastatic Colorectal Cancer.	D. Curiel	6/9/94
Clinical Trial to Assess the Safety, Feasibility, and Efficacy of Transferring a Potentially Anti-arthritic Cytokine Gene to Human Joints with Rheumatoid Arthritis.	C.H. Evans	6/9/94
Use of Safety-Modified Retroviruses to Introduce Chemotherapy Resistance Sequences into Normal Hematopoietic Cells for Chemoprotection During the Therapy of Breast Cancer: A Pilot Trial.	A. Deisseroth	6/9/94

[a]The protocols listed were approved through August, 1994. Detailed protocols for these clinical trials are published in the monthly journal *Human Gene Therapy*.

Table 5–1
Therapeutic Gene Therapy Trials Approved by the Recombinant DNA Advisory Committee of the National Institutes of Health.* (*Continued*)

PROTOCOL TITLE	PRINCIPAL INVESTIGATOR	DATE OF APPROVAL
Retroviral Mediated Gene Transfer of the Fanconi Anemia Complementation Group C Gene to Hematopoietic Progenitors of Group C Patients.	J.M. Liu	6/9/94
Clinical Protocol for Modification of Tumor Suppressor Gene Expression and Induction of Apoptosis in Non-Small Cell Lung Cancer (NSCLC) with an Adenovirus Vector Expressing Wildtype p53 and Cisplatin.	J.A. Roth	6/10/94
Infection of Glioblastoma Patients with Tumor Cells Genetically Modified to Secrete Interleukin-2 (IL-2): A phase I Study.	R.E. Sobol	6/10/94
IL-2 Gene Therapy Using Direct Injection of Tumor with Genetically Engineered Autologous Fibroblasts.	M.T. Lotze	6/10/94
Phase I/II Study of Autologous Human GM-CSF Gene Transduced Prostate Cancer Vaccines in Patients with Metastatic Prostate Carcinoma.	J. Simons	8/3/94

*The protocols listed were approved through August, 1994. Detailed protocols for these clinical trials are published in the monthly journal *Human Gene Therapy*.

that directly contributes to the disorder, or a gene that mediates an unrelated biochemical process, may be the basis for intervention. This diversity of approaches in treating acquired illnesses is illustrated in the gene therapy strategies that have been proposed for treating AIDS and various cancers. Treatment of HIV infection potentially could rely on the interruption of viral processes that directly contribute to the pathogenesis of AIDS. This could be achieved by several means, including inserting a gene that produces antisense mRNA, catalytic RNA (ribozymes), or a dominant negative mutant protein.

Vaccination. Gene transfer-mediated vaccination has become a rapidly expanding field and is applicable to the treatment of both noninfectious and infectious diseases.

Vaccination Against Noninfectious Diseases. Gene therapy for neoplastic diseases includes efforts to engineer an immune response to tumor cells. The idea that tumor cells can be used to elicit an antitumor immune response is founded in rare clinical observations of spontaneous tumor regression, the fact that some tumors are more common in immunocompromised hosts, and the discovery of tumor-associated antigens on many different tumor types. The general strategies proposed include transducing autologous tumor cells (or tumor infiltrating lymphocytes) to secrete a specific cytokine (*e.g.*, tumor necrosis factor, interleukin-2, interleukin-4, interferon gamma, *etc.*), inducing tumor cell expression of a strong rejection antigen (*e.g.*, allogeneic major histocompatibility or MHC molecules), and inducing tumor cell expression of lymphocyte costimulatory molecules (*e.g.*, B7-1). Several of these approaches have reached the stage of clinical trials, but the data from these phase I studies are limited and insufficient to indicate their therapeutic effectiveness (for reviews of this topic, *see* Nabel *et al.*, 1994).

Vaccination Against Infectious Diseases. The use of gene transfer to stimulate immunity to infectious agents also is under investigation. Insertion of DNA sequences that encode key antigens from pathogenic agents (subunit vaccines) would allow for the cellular synthesis and presentation of these antigens in a manner that physiologically mimics their presentation during infections, without the risks of actual exposure to the pathogenic organism. This could have significant implications in the development of an HIV vaccine where the safety implications of a live, attenuated HIV vaccine are awesome.

Obstacles to Gene Therapy

The therapeutic applications of gene transfer technology increase with each discovery of a new cellular process. At present, our ability to develop clinically efficacious therapies from scientifically sound principles is limited by several problems that, to some extent, plague all gene therapy strategies. For the foreseeable future, gene therapy is limited to somatic cells (nongerm-line cells). How these cells in a given tissue are targeted by the DNA delivery method has been an area of intense interest. Once the gene has been successfully transferred, the duration of transgene expression becomes important. Finally, the DNA vector itself must be analyzed for its potential to cause unwanted side effects (Jolly, 1994).

DNA Delivery and Pharmacokinetics. The delivery of exogenous DNA and its processing by target cells require the introduction of new pharmacokinetic paradigms beyond those that describe the conventional medicines in use today (*see* Chapter 1). With *in vivo* gene transfer, one must account for the fate of the DNA vector itself (volume of distribution, rate of clearance into tissues, *etc.*), as well as for the consequences of altered gene expression and protein function. A multicompartmental model to describe these events in a quantitative fashion has been developed

(Ledley and Ledley, 1994). Processes that must be considered include the distribution of the DNA vector following *in vivo* administration; the fraction of vector taken up by the target cell population; the trafficking of the genetic material within cellular organelles; the rate of degradation of the DNA; the level of mRNA produced; the stability of the mRNA produced; the amount and stability of the protein produced; and the protein's compartmentalization within the cell, or its secretory fate, once produced. It is conceivable, although yet to be realized, that each of these events may be incorporated into the design of the gene transfer system in a rational way so as to tailor the gene transfer to the specific requirements of the disease being treated.

Duration of Expression of Transferred Gene. The length of time over which the transferred gene will function is of tremendous importance. In the treatment of inherited diseases, it would be desirable to have stable gene expression over many years. In contrast, in the treatment of malignancy, it is possible that the long-term production of the therapeutic protein could have deleterious consequences. Durable gene expression has yet to be conclusively demonstrated by any of the human trials to date, but this relates as much to the short term of follow-up as to experimental design. Vectors that integrate the transferred DNA into the chromosomes of the recipient cell have the greatest potential for long-term expression. Retroviral vectors and adeno-associated viral vectors have integrating functions. The persistence of the transgene DNA in the DNA of the recipient cell does not, however, guarantee long-term gene expression in that cell. The production of the intended mRNA and protein may decline due to inactivation of the transgene promoter even though the DNA persists. In some circumstances, loss of transgene expression may occur due to loss of the transduced cell by host immune processes (*see* Jolly, 1994, for detailed discussion of this issue).

Adverse Consequences of Heterologous Gene Expression. Along with factors that limit gene transfer and expression, there is a growing list of adverse consequences that may arise as a result of successful gene transfer. As with any new drug, it will be impossible to predict these events in advance of more clinical experience. Nonetheless, some specific events can be anticipated independent of the transgene employed. Because, in most circumstances, gene transfer will result in the synthesis of a new protein, the possibility of an immune response must be considered. A severe immune response could inactivate a secreted product (as is seen in hemophilia patients receiving factor VIII replacement therapy) or lead to an "autoimmune" response to transduced tissues. In some circumstances, the DNA vector itself may be immunogenic, as has been demonstrated for adenovirus vectors. An immune response to the vector may preclude its readministration or limit the duration of its effectiveness.

Pathological events may arise from viral vector replication. Significant efforts have been directed toward the design of viral vectors that are unable to replicate (replication-incompetent) in the target cell. This has been achieved by the deletion of specific genes from the viral genome that are necessary for viral replication (Miller *et al.*, 1993; *see also* legends to Figures 5–1 and 5–2). In order to produce the virus, it must then be grown *in vitro* in a cell specifically designed to provide those functions removed from the virus. By these means, replication-defective retroviruses, adenoviruses, adeno-associated viruses, and herpes viruses have been produced. This approach does not completely eliminate replicative potential in all circumstances. The virus may overcome the deletion of replication machinery by the use of unidentified host cell factors or by recombination in the patient with wild-type viruses. Fortunately, in the limited patient experience to date, these events have not been reported.

Ethical Issues

As with any new technology, much attention has been directed toward ethical issues of gene therapy. Many of these issues are common to all new and expensive forms of medical treatment, such as who will have access to the therapy, and who will pay for it. The perception that this technology could be used for germ-line genetic engineering has spawned much discussion as well (Neel, 1993). Also of concern is the possibility that gene transfer techniques would be used for "frivolous" purposes such as cosmetic alterations. While these issues likely will be topics of continued debate, they, at present, deal with very unlikely events. For example, gene transfer into germ-line tissues to prevent future generations of affected children would require "prophylactic" treatment of prospective parents. Since the risk of having an affected child in the vast majority of cases is either one in two (autosomal dominant disease), or one in four (autosomal recessive disease), and the treatment will be neither without risk nor 100% effective, it is unlikely that any reasonable parent would submit to such a procedure. Even if there were successful introduction of a new gene during the process of *in vitro* fertilization, it is unlikely that the corrected phenotype would persist for more than one generation. The new gene would have to be inserted into the same chromosome (23 to 1 odds against this), and in close proximity to the de-

fective gene (100 to 1 odds against this), so that the new gene would be tightly linked to the defective gene. Alteration of normal characteristics is even more farfetched, as we have only a primitive understanding of the many factors that control physical appearance, personality, intelligence, and physical ability, and the genetic contribution to these characteristics.

TECHNOLOGIES FOR *in vivo* GENE TRANSFER

The ideal DNA delivery system would be one that could accommodate a broad size range of inserted DNA, was available in a concentrated form, was easily produced, could be targeted to specific types of cells, would not permit replication of the DNA, could provide long-term gene expression, and was nontoxic and nonimmunogenic. Such a DNA delivery system does not exist, and none of the available technologies for *in vivo* gene transfer is perfect with respect to any one of these points. As of 1995, three gene transfer systems (retroviral vectors, adenoviral vectors, and liposomes) had been used in human gene therapy trials, with a total clinical experience of a few hundred patients worldwide. Consequently, the following discussion will highlight conceptual strategies and issues to be refined, rather than clinical experience.

Viral Vectors

The natural life cycle of mammalian viruses has made them a logical starting point for the design of therapeutic gene transfer vehicles, because viruses all transfer and express exogenous genetic material during infection. In the simplest analysis, a virus consists of genetic material encapsulated in a particle that can be taken up by the target cell, leading to the expression of virally encoded genes. For viral vectors to be useful, several viral functions must be altered. At a minimum, the virus must be rendered replication-incompetent to prevent uncontrolled spread of the transgene and must have some element of its own genome removed to allow for insertion of the transgene. Beyond this, additional modifications are dependent on the specific virus. Viral vectors have been used extensively in preclinical research and are the basis for the majority of gene therapy clinical trials now underway.

Retroviruses. Retroviral vectors have had the greatest clinical use so far and offer the potential for long-term expression from a stably integrated transgene. They lack irrelevant and potentially immunogenic proteins, and there is no preexisting host immunity to the vector. Their application, however, is limited to dividing cells. Large-scale production is technically possible, although purification and concentration potentially are problematic due to the instability of the virus. Several safety issues have been raised but have not as yet been supported by clinical experience.

Retroviruses were first described for gene transfer applications in 1981 and first utilized in clinical trials in 1989 (Rosenberg *et al.*, 1990). Retroviruses are composed of an RNA genome that is packaged in an envelope derived from host cell membrane and viral proteins. For the retrovirus to effect gene expression, it must first reverse transcribe its positive-strand RNA genome into double-stranded DNA, which is then integrated into the host cell DNA. This process is mediated by reverse transcriptase and integrase proteins contained in the retrovirus particle. The integrated provirus is able to use host cell machinery to carry out transcription of viral mRNAs and their subsequent processing and translation into viral proteins. The virus completes its life cycle by synthesizing new positive-strand RNA genomes from the integrated provirus. An encapsidation signal (ψ) within the RNA mediates the organization of the viral genomic RNA and proteins into particles that bud from the cell surface.

Design of the Retroviral Vector. The genomic organization of retroviruses is simple, and this property facilitates its manipulation into vectors for use in gene therapy. The murine leukemia virus and its congeners are the most widely used retroviral vectors (Miller *et al.*, 1993). Retroviral vectors are constructed from the proviral form of the virus. The *gag*, *pol*, and *env* genes are removed to make room for the gene(s) of therapeutic interest and to eliminate the replicative functions of the virus (*see* Figure 5–1 for a strategic overview). Up to 8 kilobases of heterologous DNA can be incorporated into the retroviral vector. Because all virally encoded mRNAs are eliminated from the recombinant retrovirus, no viral proteins are produced by retroviral vectors. This removes any potential viral-encoded antigens that might lead to an immune response to the transduced cells. Along with the gene of therapeutic interest, genes encoding antibiotic resistance often are included in the recombinant retrovirus as a means of selecting the virus-harboring cultured cells *ex vivo*. The bacterial gene for aminoglycoside-3′-phosphotransferase, which confers resistance to kanamycin, neomycin, and geneticin, and the gene for hygromycin B phosphotransferase, which confers resistance to hygromycin, are two such examples of antibiotic resistance genes introduced into retroviral vectors for gene therapy. The presence of an antibiotic-resistance gene facilitates isolation of the recombinant retrovirus and subsequent determination of virus titer. Sequences containing promoter and enhancer functions also may be included with the transgene to facilitate its efficient expression and, in some circumstances, to provide for tissue-specific expression after administration *in vivo*. Alternatively, the promoter and enhancer functions contained in the long terminal repeat of the virus may be used for this purpose.

Packaging Cell Lines. After deletion of the genes encoding viral structural proteins and proteins that mediate viral replication, these viruses can be produced only in specially engineered viral packaging cell lines that are capable of providing these proteins (*see* Figure 5–1). The packaging cell line is optimally constructed by stably inserting the deleted viral genes (*gag*, *pol*, and *env*) into the cell in such a manner that these genes will reside on different chromosomes within the packaging cell. This strategy ensures that recom-

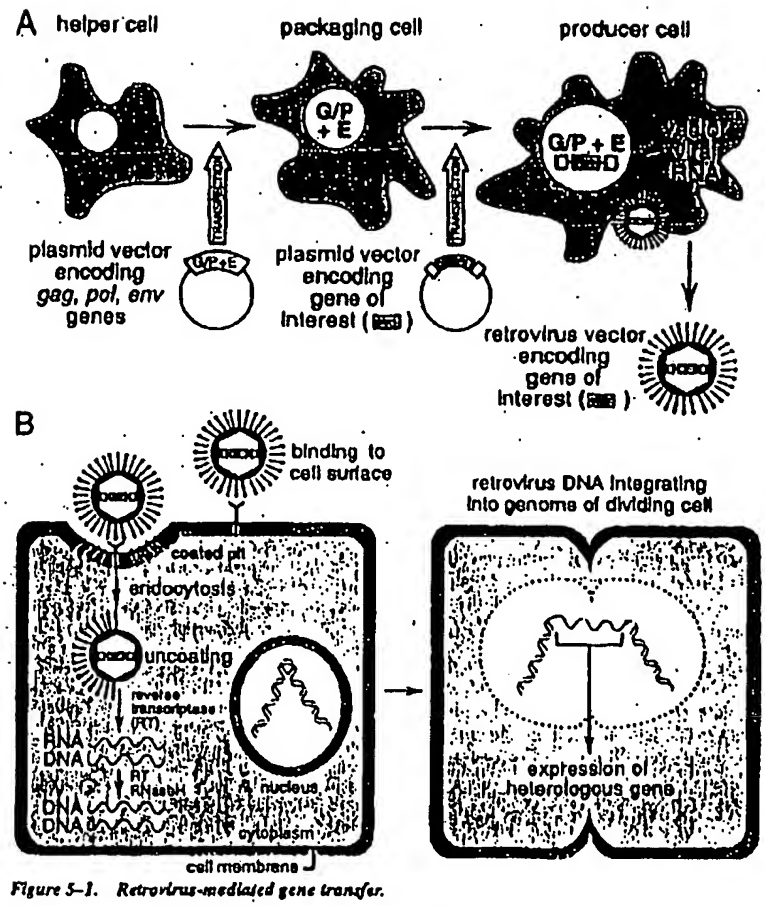


Figure 5–1. Retrovirus-mediated gene transfer.

A. Overall strategy of retroviral production. Replication defective retrovirus vectors are produced from a helper cell that is engineered to provide viral functions (DNA) which have been removed from the virus. The *gag* (G), *pol* (P), and *env* (E) DNA sequences are cloned into DNA plasmids which are then transfected into the helper cell to produce the *packaging cell.* Packaging cells are able to produce the gag, pol, and envelope proteins required for retroviral replication. A plasmid containing recombinant proviral DNA, but lacking *gag, pol,* and *env* genes, is transfected into the packaging cell line to create the *producer cell* which contains all of the molecular machinery necessary to reproduce the recombinant retrovirus that is secreted into the tissue culture medium. Only the recombinant proviral sequence is packaged into the retrovirus. Because the recombinant retrovirus does not contain the *gag, pol,* and *env* genes, cells that this replication-defective recombinant retrovirus infects cannot produce additional virions.

B. Expression of gene of interest in target cell following retrovirus-mediated RNA delivery.

bination of these genes is highly unlikely. In the absence of such a recombination, it is impossible to produce an intact viral genomic RNA that could be packaged into a replication-competent virus. The packaging cell line is used to construct a retroviral producer cell line that will generate replication-defective retrovirus containing the gene(s) of interest. This is done by inserting the recombinant proviral DNA into the packaging cell line. The recombinant proviral DNA is in the form of plasmid DNA containing the long terminal repeat sequences flanking a small portion of the *gag* gene that contains the encapsidation sequence and the genes of interest. This is transfected into the packaging cell line using any number of standard techniques for DNA transfer and uptake (electroporation, calcium precipitation, *etc.*). Several versions of this basic design have been employed to decrease the likelihood of recombinant events that could lead to the production of replication-competent virus (Jolly, 1994). Additional modifications have been employed to alter the host cell range of the virus. This is determined to a large extent by the envelope gene (*env*). The Moloney murine leukemia virus envelope is ecotropic, which means that infection is restricted to the cells of a particular species, in this case mouse. An envelope affording a broader infection range is available by using the *env* gene from the 4070A strain of murine leukemia virus. This envelope gene has amphotropic specificity and can promote the infection of human, murine, and other mammalian cells. *Env* genes with specificities that extend the host range to nonmammalian cells also are available. Efforts to design new ligands into the envelope protein have met with limited success, as the virus produced often is of low titer. Nonetheless, the ability to specifically target the virus by redesign of its molecular structure is an important goal and undoubtedly will receive more attention in the future.

Clinical Administration of Retroviruses. The clinical administration of retroviruses has been accomplished by the *ex vivo* transduction of patients' cells, by the direct injection of virus into tissue, and by the administration of the retroviral producer cells.

Ex Vivo Gene Transfer. The *ex vivo* approach has been most widely employed in human clinical trials. Although cumbersome in that it requires the isolation and maintenance in tissue culture of the patient's cells, it has the advantage that the extent of gene transfer can be quantified readily and a specific population of cells can be targeted. In addition, a high ratio of viral particles to target cells can be achieved and thus improve the transduction efficiency. This approach was used to modify lymphocytes (Anderson *et al.*, 1990; Rosenberg *et al.*, 1990; Culver *et al.*, 1991) and hematopoietic cells (Nienhuis *et al.*, 1991), in the treatment of adenosine deaminase deficiency (Anderson *et al.*, 1990), in the treatment of hyperlipidemia (Grossman *et al.*, 1994) (*see* Figure 5–4, below), and to express immune modulatory agents in tumor cells (Lotze *et al.*, 1992; Lotze, 1993; Lotze *et al.*, 1994). Clearly, not all potential disease applications are amenable to *ex vivo* gene transfer, as the removal and culture of patient cells may not be technically possible. In such circumstances, direct introduction of the virus *in vivo* is necessary.

In Vivo Gene Transfer. Retroviruses are being tested as potential agents to treat brain tumors which, in many circumstances, are relatively inaccessible. Here, the inherent ability of a retrovirus to transduce only dividing cells (tumor cells) and leave nondividing cells (normal brain parenchyma) untouched may be particularly advantageous. Although the direct stereotactic injection of recombinant retrovirus into the target tissue is possible, the efficiency of gene transfer generally is very low.

Several factors contribute to the inefficiency of retroviral gene transfer *in vivo*. Retrovirus preparations are relatively dilute compared with other vectors, typically with 10^6 to 10^8 plaque forming units per milliliter. Furthermore, the virus can transduce only dividing cells, and within the target tissue only a small fraction of cells may be dividing in the time interval between virus injection and virus clearance. Thus, even with a large excess of virus, only a fraction of the cells are effectively transduced. To overcome these difficulties, Oldfield and colleagues (1993) proposed the administration of a retrovirus producer cell line directly into patients' brain tumors using stereotactic injection. Their hypothesis was that the murine producer cell would survive within the brain tumor for a period of days, and that over this time period would secrete retrovirus capable of transducing the surrounding brain tumor. Studies are in progress in a limited number of patients using virus carrying the herpes virus thymidine kinase gene. This gene renders the cells susceptible to killing by the systemically administered antibiotic ganciclovir, which is metabolized to a cytotoxic metabolite by thymidine kinase. Several important issues will need to be addressed before this approach gains widespread acceptance. The ability of the virus to diffuse from the producer cell to nonneighboring tumor cells is not yet well quantified. If the area of transduced tumor cells is small, tumor cells lying in microscopic cords of tumor infiltrating normal brain might go untreated. Also unknown is whether an immune response to the xenogeneic producer cell line precludes subsequent retreatment of residual tumor. This will be very important given that, over the time of virus secretion, all tumor cells may not be actively dividing, and therefore some cells might go unscathed. Serial treatments, as in conventional chemotherapy, might be required to achieve complete tumor eradication. The results of clinical trials now under way and subsequent studies may answer these questions.

Safety of Retroviral Vector Strategies. The use of retroviral vectors has raised several important safety issues. One concern is that because the virus integrates into the target cell chromosomes (an attractive feature for long-term expression) and because integration occurs in a nearly random fashion, integration could be mutagenic. For example, undesired mutations might occur if insertion of the retroviral DNA altered the function of a cell growth regulating gene. Although replication-competent retroviruses have tumorigenic potential, this has not been observed with the replication-defective vectors that are in use as gene transfer agents. Additionally, this has not been observed in any patients who have received retroviral gene therapy. However, the number of patients studied to date is too few and their follow-up too short for current clinical experience to be extrapolated to long-term safety.

Demonstrating that retroviral agents are free of replication-competent virus is of paramount importance. Replication-competent virus could arise by several means. As noted earlier, recombination of the retroviral genetic elements inserted into the packaging cell line is exceedingly unlikely. Recombination with other retroviral genomes is, however, theoretically possible. There exist homologous endogenous retroviral sequences within the mouse cell lines used to create packaging cell lines. The use of dog- or human-derived packaging cell lines that are free of such sequences has been proposed (Jolly, 1994). Recombination with retroviral sequences in the target cell is theoretically possible. Wild-type murine retroviruses, from which genetic vectors are derived, do not infect human cells. Therefore, it is unlikely that a wild-type virus could infect the same target cell and lead to rescue of the defective retroviral vector. However, there do exist endogenous retroviruses in all human tissue (HERV-K retroviruses)

that have low-level homology to the retroviral vectors. It is very unlikely that this type of recombination would occur with sufficient frequency to lead to clinically significant adverse outcomes. In the final analysis, the safety of these and other vectors must be determined by direct clinical experience and their safety weighed against their therapeutic benefits.

Adenoviruses. Over 40 serotypes of human adenoviruses are known, and many animal adenoviruses have been characterized to varying degrees. The clinical spectrum of human adenoviral infections is well described (*see* Horwitz, 1990). Infections involving the respiratory tract are common and typically self-limited in normal hosts. Gastrointestinal, urinary, hepatic, and CNS infections occur sporadically. Most, if not all, adults have prior exposure to adenovirus and are seropositive for antiadenovirus antibodies when tested by sensitive methods. In the United States, military recruits specifically are vaccinated with a polyvalent adenoviral vaccine to prevent outbreaks of respiratory infections (Rubin and Rorke, 1994). In contrast to the retroviruses, these larger, nonenveloped viruses possess a double-stranded DNA genome, and replicate independent of host cell division.

Adenoviral vectors possess several attractive features that have encouraged their development for clinical use. They are capable of transducing a broad spectrum of human tissues, including respiratory epithelium, vascular endothelium, cardiac and skeletal muscle, peripheral and central nervous tissue, hepatocytes, the exocrine pancreas, and many tumor types. Exceptionally high levels of gene transfer and transgene expression can be obtained in dividing and nondividing cells. Several routes of administration can be used including intravenous, intrabiliary, intraperitoneal, intravesicular, intracranial and intrathecal injection, and direct injection of the target organ parenchyma. So far it has not been possible to modify the adenovirus to achieve a tissue-specific virus. The multiple routes of administration may overcome this deficiency by providing flexibility in targeting based on anatomical boundaries.

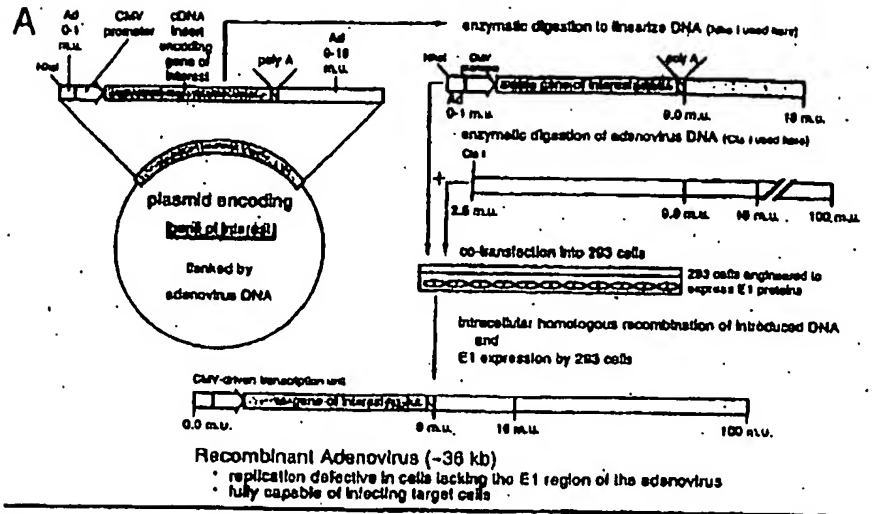
Clinical trials using adenovirus have been limited to date to the ongoing protocols for cystic fibrosis, where the recombinant adenovirus is delivered by aerosolization into the respiratory tract. Studies using direct administration of adenoviral vectors into the liver to treat inherited genetic deficiencies and into a variety of tumors likely will begin in the near future (*see* Ohno *et al.*, 1994, and Kozarsky *et al.*, 1994, as two examples of adenoviral gene therapy strategies).

The genomic structure of adenoviruses is more complex than that of retroviruses. The adenoviral genome encodes approximately 15 proteins. Infection takes place when the fiber protein, which extends from the icosahedral capsid, binds a cell surface receptor. Subsequently, peptide sequences in the penton base portion of the capsid engage integrin receptor domains ($\alpha_v\beta_5$, or $\alpha_v\beta_3$) on the cell surface. This leads to virus internalization via endosomal pathways where the viral particle begins to disassemble. The virus escapes the endosome prior to its fusion with lysosomal compartments and thus avoids digestion. The viral DNA is able to enter the target cell nucleus and begin transcription of viral mRNA without concomitant cell division. Although integration of viral DNA into the host cell genomic DNA can occur at high levels of infection in dividing cells, this is a relatively infrequent event and does not contribute significantly to the utility of these viruses as vectors. Viral gene expression and replication occur in an ordered fashion and are driven in large measure by the E1A and E1B genes in the 5' portion of the adenoviral genome. The E1A and E1B genes provide transactivation functions for transcription of several of the downstream viral genes (*see* Horwitz, 1990).

Since the E1 genes are involved intimately in adenovirus replication, their removal renders the virus replication-incompetent or, at the very least, severely crippled with respect to replication. Due to the complexity of the virus, it has been more difficult to remove all adenoviral genes as is done with retroviral vectors. The expression of adenoviral proteins, with the currently employed adenoviral vectors, leads to both a cellular and a humoral immune response to recombinant adenoviral vectors. In some instances, this may limit the utility of this vector both in terms of host immune response to adenovirally transduced cells and with respect to readministration of the vector.

Design of Adenoviral Vectors for Gene Therapy. Although several adenoviral serotypes are known, serotypes 2 and 5 have been most extensively used for vector construction. Adenoviral vectors can be constructed using one of several general approaches. A schematic diagram outlining the basic elements of an adenoviral vector design for gene therapy is shown in Figure 5–2. Bett and colleagues (1994) have developed an adenoviral type 5 vector system based on bacterial plasmids containing the adenovirus genome with deletions of the E1 and E3 adenoviral genes. Deletion of E1 renders the virus replication-defective. In addition, all or part of the E3 region, which is not essential for virus function, is deleted in order to accommodate the DNA inserted into the adenovirus genome. Genes of interest can be cloned into the deletion regions, and the plasmid vector can then be grown in bacterial culture. The purified plasmid DNA subsequently is transfected into the 293 line of human embryonic kidney cells. The 293 cell line has been engineered to express E1 proteins and can thus transcomplement the E1-deficient viral genome. The virus can be isolated from 293 cell media and purified by limiting dilution plaque assays (Graham and Prevek, 1991). An alternative approach is to prepare a plasmid containing the gene of interest, flanked by adenovirus DNA sequences. Transfection of this plasmid into 293 cells along with genomic adenovirus DNA with selected deletions (*e.g.*, E3) leads to formation of adenoviral particles with the transgene replacing E1 genes by homologous recombination. It is this strategy that is given in detail in Figure 5–2. Either direct cloning or homologous recombination can be used to produce E1-deleted, replication-defective adenovirus.

Large amounts of the engineered adenoviral vector system can be produced by growing the recombinant virus in 293 cell cultures. The virus is isolated by lysing the infected 293 cells and purifying the crude lysate by cesium chloride density centrifugation, a procedure that not only separates the virus from other tissue culture–derived substances, but also concentrates the virus to very high titers (over 10^{13} particles per ml). The purified virus is remarkably stable in a variety of aqueous buffers, and can be frozen for a prolonged period of time without loss of activity.



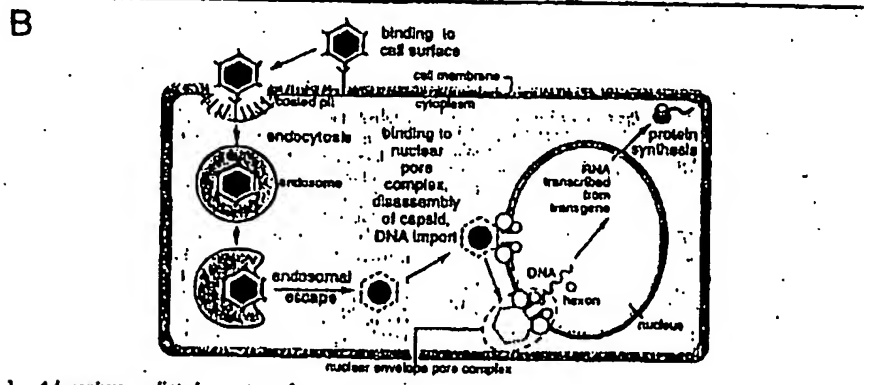


Figure 5–2. Adenovirus-mediated gene transfer

A. Construction of recombinant adenovirus for engineering cells. Strategy for preparing recombinant adenovirus by homologous recombination. Recombinant adenovirus encoding a gene of interest can be produced by cloning the gene of interest (shown in blue) into a plasmid. This transgene is flanked by a promoter sequence (*e.g.*, CMV promoter) and by regions of the adenovirus genome (shown in gray). The example here is based on adenovirus 5. The adenovirus 5 DNA is divided into 100 map units (m.u.; 360 base pairs per map unit). Deletions are made in the adenovirus DNA to remove E1 (1 to 9.2 m.u.) and E3 (78.4 to 84.3 m.u.) regions to eliminate the possibility of autonomous replication and to allow room for insertion of the transgene. Homologous recombination takes place between the plasmid DNA and the adenovirus genomic DNA to yield the recombinant virus. Since the transgene sequence replaces the E1 genes of the adenovirus, the adenovirus is unable to replicate in cells other than those engineered to express E1 gene products, such as the human embryonic kidney 293 cells shown here.

After linearization of the plasmid by digestion with an endonuclease (*e.g.*, Nhe I in this example), the transgene-expressing plasmid is cotransfected with adenovirus genomic DNA from which the 3′ end has been removed (*e.g.*, digestion with the Cla I endonuclease at Ad 2.5 m.u.), also to prevent autonomous adenovirus replication, until homologous recombination takes place, which in this example occurs within the 293 cells.

B. Adenovirus-mediated infection of target cells. Expression of gene of interest in target cell following adenovirus-mediated DNA delivery. A recombinant adenovirus binds to specific receptors on the surface of a target cell and enters the cell by endocytosis. Viral proteins promote the escape of the adenovirus from the endosome prior to endosome fusion with and destruction by lysosomes. The adenovirus DNA becomes unpackaged from the viral proteins and travels to the nucleus where it begins to synthesize new mRNA. The adenovirus encoded DNA, including the transgene, is not integrated into the genome of the host cell. (*Modified from Graber et al., 1993, with permission.*)

Duration of Transgene Expression. Adenoviral vectors currently are limited by their relatively short duration of transgene expression. Several factors contribute to this, including clearance of transduced cells by cytotoxic T cells and other inflammatory cells (Yang *et al.*, 1994) and dilutional loss of episomal DNA during target cell division. The former likely will be solved by the design of adenoviral vectors that are less immunogenic. Vectors with temperature-sensitive mutations in the E2 region clearly are less immunogenic and offer significantly longer gene expression (Engelhardt *et al.*, 1994). Deleting the E4 gene from adenoviral vectors also may diminish the immune response to transduced cells (Armentano *et al.*, 1994). Subsequent generations of adenoviral vectors with additional modifications of the adenoviral genome or the use of nonhuman adenoviruses may advance the use of adenoviral vectors. The episomal nature of the adenovirus genome ultimately limits the duration of gene expression in tissues with active cell division such as bone marrow and epithelial surfaces. Since each round of target cell division after gene transfer is not accompanied by replication of the transgene, daughter cells will have progressively fewer and eventually no copies of the transgene. Integration of the adenoviral vector does occur, but not at a high enough frequency to be useful.

Safety of Adenoviral Vector Strategies. The safety of adenoviral vectors likely will be borne out by current clinical trials. The principal side effects are from the host immune response to the adenoviral proteins, a limitation that may be eliminated by future generations of vectors. There is some concern, however, that vector replication can take place despite removal of important regulatory genes. Since wild-type adenoviral infections are common, there exists the possibility that wild-type viruses may recombine with replication-defective vectors to produce replication-competent, recombinant virus. Although not observed in the present cystic fibrosis clinical trials, this remains a concern. Additionally, there is a growing body of evidence that some cell types may contain proteins with functions homologous to E1a and thus be able to provide a permissive environment for recombinant viral replication. With the present adenoviral vectors, this is not likely to evolve into a serious infection, given the preexisting host immunity to adenoviral infection. However, if future adenoviral vectors are able to evade this protective mechanism, then recombinant viral replication may become a greater concern.

Adeno-Associated Virus. Adeno-associated virus (AAV) appears to have many of the desirable features of retroviruses and adenovirus without some of their potential drawbacks for application to gene therapy (Kotin, 1994). These single-stranded DNA, nonautonomous parvoviruses are able to integrate efficiently into the genome of nondividing cells of a very broad host range. Integration of the wild-type virus is specific for chromosome 19 (19q13.3-qter), or at least shows preferential integration at this site. Although ubiquitous in nature, AAV has not been shown to be associated with any known human disease and does not elicit an immune response in an infected human host. AAV is a nonenveloped virus that is stable to a variety of chemical and physical manipulations and thus can be purified, concentrated, and stored for prolonged periods.

At present, the use of AAV as a vector for gene therapy is limited by difficulties in producing the virus in large quantities and, more importantly, by a lack of understanding of the biology of the recombinant virus. For instance, it remains to be determined whether or not these vectors have the ability to infect and integrate into nondividing cells, an important feature of the wild-type virus that has promoted its use. There is little experience in human beings with these new vectors. The Recombinant DNA Advisory Committee of the National Institutes of Health has approved the first human trial of AAV in patients with cystic fibrosis. This trial may provide information about the duration of gene expression following AAV-mediated gene transfer into terminally differentiated airway epithelial cells.

AAV has two distinct phases to its life cycle. In the absence of helper virus (adenovirus), the wild-type virus will infect a host cell, integrate into the host cell genome, and remain latent for a long time. In the presence of adenovirus, the lytic phase of the virus is induced, which is dependent on the expression of early adenoviral genes, and leads to active virus replication. Structurally, the AAV genome is composed of two open reading frames (called *rep* and *cap*) flanked by inverted terminal repeat (ITRs) sequences. The *rep* region encodes four proteins which mediate AAV replication, viral DNA transcription, and endonuclease functions used in host genome integration. The *rep* genes are the only AAV sequences required for viral replication. The *cap* sequence encodes structural proteins that form the viral capsid. The ITRs contain the viral origins of replication, provide encapsidation signals, and participate in viral DNA integration. The function of many of these proteins and the overall biology of the virus have been studied largely in wild-type viruses (*see* Kotin, 1994). Recombinant, replication-defective viruses that have been developed for gene therapy lack *rep* and *cap* sequences. The recombinant viruses are less well studied, and it is not known whether these viruses retain all of the features of the wild-type virus (*i.e.*, site-specific integration in a nondividing cell).

Production of AAV in large quantities is considerably more difficult than production of retroviruses or adenoviruses. Replication-defective AAV can be produced by cotransfecting the separated elements necessary for AAV replication into a permissive cell line (typically 293 cells). In a commonly used method, plasmid DNA containing *rep* and *cap*, under the control of AAV promoters but lacking ITRs, is transfected into 293 cells. DNA containing the gene to be "packaged" (promoter, enhancer, transgene, polyadenylation signal) flanked by ITRs is cotransfected at the same time. Infection with adenovirus provides helper functions that induce the synthesis of rep proteins, which in turn transactivate the synthesis of capsid proteins. The transgene flanked by ITRs is then packaged into viral particles that can be isolated and purified by cesium chloride density centrifugation. This approach requires that the plasmid expressing the ITR (ITR^+; here, the transgene-encoding plasmid) have little sequence homology with ITR^- plasmids (*cap* and *rep*) to reduce the likelihood of recombination events that could lead to the production of wild-type virus. Improved systems for recombinant AAV preparation are being developed including the use of producer cell lines that provide rep and cap functions. Such an approach not only would simplify the transfection scheme, but also would provide rep and cap proteins in larger quantities and thus lead to higher yields of recombinant virus.

Vaccinia Vectors (Pox Viruses). The extensive clinical experience with vaccinia vaccines and their ease of ma-

nipulation have led to efforts to develop gene therapy vectors from pox viruses (Moss and Flexner, 1987; Moss, 1990). Vaccinia are large, enveloped DNA viruses that replicate in the cytoplasm of infected cells. Like adenovirus, they can infect nondividing as well as dividing cells from many different tissues, and provide short-term gene expression from a nonintegrated viral genome. Recombinant virus can be produced by inserting the transgene into a vaccinia-derived plasmid and transfecting this DNA into vaccinia-infected cells. Homologous recombination leads to the generation of the recombined virus that can be plaque purified. High yields of virus are achieved easily and can be stored for long periods of time. The vaccinia viruses can accommodate much larger DNA inserts than can retrovirus, adenovirus, or AAV vectors. Additionally, since the wild-type virus no longer exists in the wild, recombination to produce new strains of virus is unlikely. A significant drawback to the use of this vector system is that it elicits a host immune response to the 150 to 200 virally encoded proteins. This is likely to make repeated administration problematic. Replication of the vector also is a concern, as it can result in significant morbidity in immunodeficient hosts. This might be overcome with newer generations of engineered vaccinia virus. At present, this vector system has not been adopted for clinical trials of human gene therapy, although it may be useful as a vaccine vector.

Herpes Simplex Virus-1 Vectors. The herpes simplex virus is a large (152 kb), double-stranded DNA virus that replicates in the nucleus of infected cells. It has a broad host cell range, and can infect dividing and nondividing cells as well as persist in a nonintegrated state. Large sequences of foreign DNA can be inserted into the viral genome by homologous recombination, and the replication-defective, recombinant virus can be plaque purified on transcomplementing cells (IE^+). These advantages for gene therapy strategies are countered by the difficulty in rendering viral preparations totally free of replication-competent virus and the elicitation of a potent immune response to virus-encoded proteins that are directly toxic to the cell. Despite these apparent drawbacks, advantages such as their ability to accommodate large DNA inserts (20 to 30 kb), the availability of high titer stocks, and their neurotropism have stimulated interest in developing useful herpes virus vectors (*see* Kennedy and Steiner, 1993).

Deletion of the viral thymidine kinase gene renders the herpes virus replication-defective in cells with low levels of endogenous thymidine kinase (*i.e.*, terminally differentiated, nondividing cells). In contrast, cells undergoing active cell division (*e.g.*, tumor cells) possess sufficient thymidine kinase activity to allow the thymidine kinase–negative herpes virus to replicate. This type of vector may be useful for treating intracranial tumors, as the tumor cells, but not the neurons, will selectively undergo gene transfer. Since vector replication occurs, systemic dissemination potentially can occur with this viral vector. This is much less likely in immunocompetent hosts because the host cellular immune response likely will control the spread of the virus. The use of herpes virus vectors in immunocompromised hosts, which may include some cancer patients, is potentially problematic (*see* Valyi-Nagy *et al.*, 1994).

Other Viral Vectors. The need for tissue-selective gene transfer has led to the consideration of a variety of other viruses, including HIV, the minute virus of mice, hepatitis B virus, and influenza virus, as possible vectors for gene transfer. These and other viruses may find applications based on aspects of their life cycle that result in tissue-selective gene expression or other unique features that lend themselves to specific diseases (*see* Jolly, 1994).

Comparison of Properties of Viral Vectors for Gene Therapy. Bovielsis and colleagues (1994) recently compared the usefulness of recombinant retrovirus, adenovirus, and herpes virus vectors in a rat brain tumor model using the gene coding for bacterial β-galactosidase as an indicator of gene transfer. Although their experiments did not definitely establish which vector is more efficient at gene transfer, useful distinguishing features of each vector were nonetheless noted. Following intralesional administration, the retrovirus and herpes virus vectors selectively effected gene transfer into tumor cells over neurons and other endogenous brain cells. In contrast, the adenoviral vector transduced brain tumor cells as well as neighboring normal brain parenchyma. In the case of the retroviral vector, selectivity for the tumor cell results from the virus's requirement for cell division as a prerequisite for transgene integration and expression. In the case of the herpes virus vector, the selectivity occurs as a result of differential expression of endogenous thymidine kinase in the tumor cells (very high) versus nonneoplastic cells (very low). The adenovirus showed little cell selectivity, and any preference for tumor cell expression probably was a result of the site of injection (within the tumor). Another noteworthy observation was the degree of inflammation and necrosis that occurred following gene transfer. The retroviral vector induced no significant inflammatory response, and that induced by the adenoviral vector was minimal. However, prominent inflammatory infiltrates were noted in the brain tissues following herpes virus–mediated gene transfer. Although this study suggests a useful role for the herpes virus vector in treating tumors, the clinical application of such a vector likely will be difficult. Additional measures to control replication of this human pathogen-derived vector will have to be instituted, and the consequences of a potentially

severe inflammatory response will need to be addressed. Furthermore, as Boviatsis and colleagues (1994) point out, the latency of this type of vector is unknown, and it is therefore possible that reactivation by recombination with wild-type virus (thymidine kinase positive) could occur.

Nonviral DNA Delivery Strategies

Because of the potential limitations of viral vectors, investigators have examined the use of nonviral agents to mediate cellular uptake of exogenous DNA. These DNA delivery systems, which include uncomplexed plasmid DNA, DNA-liposome complexes, DNA-protein complexes, and DNA-coated gold particles, are constructed from known components. Therefore, their composition, unlike complex virions, is well-defined. In addition, their formulation technically is much easier than that of viruses and, in many cases, these DNA delivery systems can be produced without the need for cell culture.

Purified Uncomplexed Plasmid DNA. Surprisingly, purified DNA (or mRNA) can be injected directly into tissues and results in transient gene expression. This has been best illustrated in muscle tissue, where direct injection of uncomplexed DNA is most effective. Wolff *et al.* (1990) demonstrated that purified plasmid DNA or mRNA encoding a reporter gene could mediate transgene expression following direct injection into the quadriceps muscle of a mouse. DNA injection resulted in longer gene expression (substantial gene product was seen after 60 days) than did mRNA injection (expression declined after 18 hours). The DNA likely persists as unintegrated plasmid DNA rather than in an integrated form. A direct comparison of adenoviral and retroviral vectors with injected plasmid DNA in murine muscle gene transfer revealed that all three systems were more efficient at gene transfer in regenerating muscle (cardiotoxin-induced) than in mature normal mouse muscle. In regenerating muscle, these DNA transfer systems were equally efficient, as assessed by the number of muscle fibers expressing the reporter gene. Surprisingly, in mature fibers, gene transfer by direct injection of plasmid DNA was superior to that with either of the viral vectors (Davis *et al.*, 1993). In addition, no inflammatory response was seen following direct DNA injection, whereas mild inflammation was seen with either viral vector. To date, direct injection of plasmid DNA has been shown to be highly effective only in skeletal and cardiac muscle. Its effectiveness may depend on features unique to the muscle fiber.

DNA-Coated Gold Particles. Plasmid DNA can be affixed to gold particles (approximately 1 micron in diameter) and then "shot" into superficial cells. The DNA is coprecipitated onto the gold particle and then propelled from a mylar sheet using an electric spark or pressurized gas as the motive force. This so-called gene-gun can be used to accelerate the DNA-coated particles into superficial cells of the skin (epidermis) or into skin tumors (melanomas). Gene expression lasts only a few days, which may be more a function of the cells targeted (*e.g.*, skin cells that are sloughed) than the method of delivery. In animal models, gene-gun delivery of DNA vaccines is highly effective (Fynan *et al.*, 1993). Gene-gun delivery is ideally suited to gene-mediated immunization, where only brief expression of antigen is necessary to achieve an immune response.

Because of the limited depth of DNA penetration, this technique is limited to surface cells that can be accessed directly. Furthermore, since the epidermal layers of the skin are rich in antigen-presenting cells, they are a preferred target for vaccination. The simplicity, safety, and technical ease of preparation of this DNA transfer system make its large-scale application more feasible than available viral DNA delivery systems.

Liposomes. Liposomes have been used extensively as a technology for delivering drugs experimentally to the interior of cells. The premise is that by surrounding hydrophilic molecules with hydrophobic molecules, agents otherwise impermeable to cell membranes might be escorted into the cell. Potential advantages of such a delivery system include targeting drugs to an intracellular location and reducing toxicity.

The basic challenge in *in vivo* gene therapy is to deliver a transgene, a large hydrophilic molecule, across the plasma membrane and into the nucleus where it can access the cell's transcription machinery. Liposome delivery technology appears well suited to this task, although it has not proven to be as efficient as hoped.

Liposomes are either unilamellar or multilamellar spheres that are manufactured using a variety of lipids. Their structure can be influenced by choice of lipid composition and manufacturing process. Proteins and other nonlipid molecules can be incorporated into the lipid membranes. For convenience, liposomes are classified as either anionic or cationic, based on their net negative or positive charge, respectively.

Anionic Liposomes. The first *in vivo* delivery of genes using liposomes was reported by Nicolau and colleagues (1983), who encapsulated a DNA transgene coding for insulin into anionic liposomes and injected the complex into rats. The transfected rats had increased circulating levels of insulin and decreased blood glucose concentrations.

In spite of this early success, there are significant drawbacks to the use of anionic liposomes for delivering DNA. These structures, when given intravenously, primarily target the reticuloendothelial cells of the liver, making them of little use for other cell targets. Because the substance to be delivered must be encapsulated within the liposomes, the manufacturing process is complex. Also, most DNA constructions necessary for gene therapy are large compared with the liposome, so that encapsulation efficiency is very low, probably prohibitively so for practical applications.

Various proteins can be inserted into the external layer of liposomes to alter their *in vivo* behavior, including cell-selective delivery. This approach can enable liposomes given intravenously to evade the reticuloendothelial system. Protein ligands or antibodies to cell surface molecules incorporated into the liposome surface also can target liposomes to specific cell surface receptors on desired cell populations (Wu and Wu, 1987). Although promising, these strategies have not yet been applied successfully to gene therapy.

Cationic Liposomes. Felgner and co-workers (1987) synthesized cationic liposomes and demonstrated that they would avidly and efficiently bind nucleic acids (which are anionic) by electrostatic interactions upon simple incubation of liposomes with nucleic acids at room temperature for brief periods. The DNA or RNA complexed to cationic liposomes readily entered cells in culture without perceptible injury to the cells. A diagram illustrating the presumed mechanism for cationic liposome-plasmid transfection is given in Figure 5–3.

In vivo, cationic liposomes have properties quite different from those of anionic liposomes. Intravenous injection of cationic complexes has been shown to effect transgene expression in most organs if the liposome-DNA complex is injected into the afferent blood supply to the organ. In addition, the liposome-DNA complexes can be administered by intraairway injection or aerosol to target lung epithelium. In experimental animals, neither intravenous injection nor aerosol delivery of cationic liposome-plasmid complexes appears to be toxic (Brigham *et al.*, 1989).

Cationic liposomes have been used to deliver DNA gene constructs in several experimental models *in vivo*. Nabel and colleagues (1994) delivered a foreign histocompatibility gene by direct injection of plasmid-liposome complexes into tumors and showed attenuation of tumor growth in murine models. Hyde and associates (1993) showed that cationic liposome-mediated gene transfer could correct CFTR-dependent, cyclic AMP stimulated chloride conductance to normal levels in transgenic mice homozygous for a null mutation in CFTR. Rabbits given intravenously the gene coding for the proximal enzyme in prostanoid synthesis (prostaglandin synthase) as a plasmid-cationic liposome complex produced increased amounts of endothelium-derived prostanoids in their lungs. This protected the lungs of the transfected animals from the effects of endotoxemia (Conary *et al.*, 1994).

Table 5–1 includes therapeutic goals in early stages of human application using liposome-mediated DNA delivery for gene therapy, such as delivery of foreign histocompatibility gene to tumors, delivery of the human α_1-antitrypsin gene to the nasal mucosa of α_1-antitrypsin-deficient patients and to subsegments of the lungs by fiberoptic bronchoscopy, and delivery of the CFTR gene to the nasal mucosa of patients with cystic fibrosis.

At present, liposome-mediated transfection offers a nontoxic, nonimmunogenic means to deliver DNA to a variety of tissues. Current usefulness of this strategy is limited by generally lower levels of gene transfer than can be obtained with viral vectors, although newer liposome formulations offer improved gene transfer efficiencies and better physical properties, e.g., higher concentrations of complex without aggregation. The applications for liposomes in gene therapy likely will expand as better reagents are developed, particularly those that facilitate targeting of specific cells.

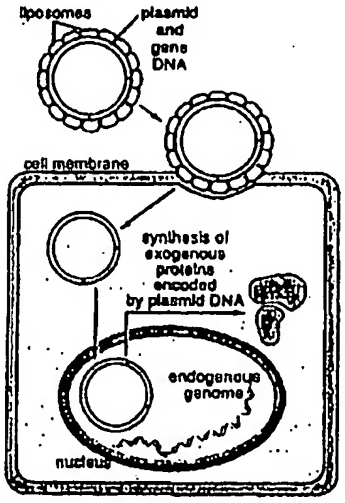


Figure 5–3. Cationic liposome-mediated DNA-delivery.

Diagrammatic representation of how cationic liposome-plasmid complexes are thought to effect gene transfer to a cell. Little is known about the actual structure of the plasmid-liposome complex. Likewise, processes affecting cell entry and transport to the nucleus are yet to be clarified. The circular plasmid DNA does not readily incorporate into the host genome and does not replicate in mammalian cells; thus transgene expression apparently is episomal in nature.

DNA-Protein Conjugates. Several groups have developed cell-specific DNA-delivery systems that utilize unique cell surface receptors on the target cell (Michael and Curiel, 1994). By attaching the ligand recognized by such a receptor to the transgene DNA, the DNA-ligand complex becomes selectively bound and internalized into the target cell (Wu and Wu, 1987). These molecular conjugate vectors are attractive because they potentially offer cell-specific gene transfer without the attendant problems of viral vectors, such as replication, immunogenic viral proteins, or recombination potential. Initial model systems focused on developing effective means of attaching the DNA to the ligand using polycations, antibody-antigen complexes, and biotin-streptavidin linkers. Poly-L-lysine (PLL), a polycation, has been widely used as it can be easily coupled to a variety of protein ligands by chemical cross-linking methods. When the PLL-ligand adduct is mixed with plasmid DNA, macromolecular complexes form in which the DNA is electrostatically bound to the PLL-ligand molecules. These toroidal structures (50 to 100 nm in diameter) present ligands to the cell surface receptor that are efficiently endocytosed. The transferrin receptor (Zenke *et al.*, 1990), the asialoorosomucoid receptor (Wu and Wu, 1987), and cell surface carbohydrates (Batra *et al.*, 1994) have been used to demonstrate the potential of ligand-mediated gene delivery. The asialo-orosomucoid receptor is of particular interest because it is found almost exclusively on hepatocytes and therefore might be useful in mediating gene transfer into the liver.

Early DNA-ligand complexes were inefficient for DNA transfer because most of the endocytosed complex was shuttled to the lysosomal compartment, and DNA was

then degraded. Although several agents (*e.g.*, chloroquine) have been used to block lysosomal degradation, the efficiency of transfection is still low compared with other DNA-delivery methods. A more effective approach is to utilize the endosomal escape functions of the adenovirus. As described earlier, proteins in the adenovirus capsid promote escape of the DNA complex from the endosome prior to fusion with the lysosome. Although metabolically inactivated adenovirus theoretically could be employed to escape lysosomal targeting, the concentrations of adenovirus required to ensure colocalization of the virus and the DNA-protein complex to the same endosome are so high as to induce adenovirus-mediated cytopathic effects. Consequently, investigators have constructed physically linked complexes between the adenovirus and the DNA-ligand adduct, thereby ensuring their simultaneous delivery to each endosome and diminishing the amount of adenovirus required to escape lysosomal delivery and degradation (*see* Fisher and Wilson, 1994).

Two general approaches have been used to construct adenovirus-DNA-ligand complexes. Poly-L-lysine can be covalently attached to purified adenoviral particles using a water-soluble carbodiimide. This is then mixed with asialo-orosomucoid receptor–poly-L-lysine–DNA toroids to form clusters of icosahedral adenoviral particles and toroids. The size of these clusters varies from small clusters (<200 nm) with single toroids coupled to two viral particles up to large clusters (200 to 300 nm) containing more than a dozen viral particles and toroids. The composition of the clusters is governed by the amount of poly-L-lysine attached to the viral particles. These complexes achieve higher levels of hepatocyte-specific gene transfer at lower concentrations of virus than do mixtures of unlinked toroids and adenovirus (*see* Cristiano *et al.*, 1993).

This technology can be further improved by layering the DNA and ligand over the surface of the adenovirus to create a coated adenovirus, rather than the side-by-side (virus-toroid-virus) structures described above (Fisher and Wilson, 1994). This creates single viral particles that retain their endosomalysis ability, are coated with DNA and extend the asialo-orosomucoid receptor from the particle surface. These smaller particles (<100 nm) still retain some adenovirus receptor recognition and uptake, similar to the larger clusters above, but their smaller size may make them better able to traverse the fenestrated hepatic endothelium. The use of two reporter genes, one carried in the plasmid DNA and the other in the adenovirus genome, has allowed the simultaneous assessment of viral infectivity and efficiency of plasmid gene transfer. By decreasing the amount of adenovirus required, virus-induced cytotoxicity essentially can be eliminated. The presence of two receptor pathways for DNA entry (ligand receptor and adenovirus receptor) clearly diminishes the specificity of this DNA delivery system. The adenovirus receptor pathway can be effectively eliminated by using an antibody against adenovirus fiber protein as the means for linkage to DNA (Michael and Curiel, 1994), an approach that obliterates the ability of the virus to bind adenovirus receptors but not its ability to mediate lysosomal escape. Further refinements, such as the use of purified endosomalytic proteins rather than intact adenovirus particles, should enhance the utility of this type of DNA-delivery system (Seth, 1994).

DISEASE TARGETS FOR GENE THERAPY

Organ-Directed Gene Therapy

Liver. Liver-directed gene therapy has emerged as an important model for the treatment of inherited and acquired disorders. The liver can be afflicted with a variety of metabolic, infectious, and neoplastic diseases for which specific molecular interventions can be envisioned. For example, gene transfer methods might be used to deliver interferon alfa for the treatment of hepatitis B, cytotoxic therapy for hepatic carcinomas, or to provide a missing gene to correct an inherited metabolic defect. Potential applications are made more feasible by the existence of multiple methods for targeting gene transfer to the liver. Molecular conjugates, adenoviral vectors, liposomes, and retroviral vectors all have been used for hepatocyte gene transfer. For *in vivo* gene transfer, the liver is accessible by a number of routes, including direct injection and intravenous and intrabiliary administration of vectors. *Ex vivo* strategies can be implemented by partial surgical resection of the liver, isolation of hepatocytes, and *in vitro* hepatocyte transduction. The genetically modified cells can be reimplanted into the liver.

Familial Hypercholesterolemia. Patients with familial hypercholesterolemia have an inherited deficiency of the low-density lipoprotein (LDL) receptor and, as a consequence, develop extremely high plasma levels of cholesterol and arteriosclerosis at a very early age (*see* Chapter 36). The genetic defect manifests itself as a diminished ability of the liver to clear LDL particles from the blood, and serum lipid levels provide a convenient marker of the disease. Although pharmacological interventions have had limited success, correction of the hepatic dysfunction by orthotopic liver transplantation leads to normalization of blood lipid levels and slowing of arterial disease progression. This clinical observation suggested that if the liver could be genetically modified to express the LDL receptor, the same benefits might be achieved. The Watanabe heritable hyperlipidemic rabbit has served as an ideal animal model to demonstrate that this approach could lead to persistent reductions in serum LDL (*see* Figure 5–4) (Chowdhury *et al.*, 1991). Several patients now have been treated in a clinical trial using an *ex vivo* DNA delivery approach and retrovirus to introduce the LDL receptor gene into hepatocytes isolated from the patients following partial hepatectomy (Grossman *et al.*, 1994). This study demonstrated the feasibility, safety, and potential efficacy of *ex vivo* hepatic gene therapy.

The overall success of DNA transfer into hepatocytes will be determined by several factors that currently are unknown. In particular, very little is known about the normal turnover of hepatocytes and how this will relate to the persistence of genetically modified cells. An immune response to the therapeutic gene product, a potential problem for all gene therapies of deficiency states, has not been observed to date. The potential for the therapeutic gene product to serve as a neoantigen may vary among different types of deficiencies and depend on the nature of the protein product and whether the deficiency arises from total absence of the protein or from expres-

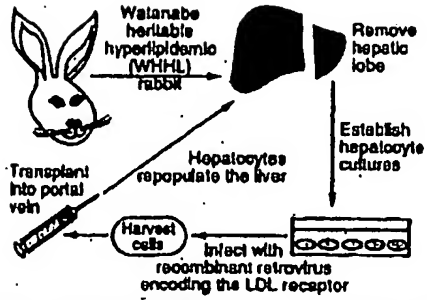


Figure 5–4 An animal model for ex vivo retrovirus gene transfer of the low density lipoprotein (LDL) receptor.

The Watanabe heritable hyperlipidemic (WHHL) rabbit is an ideal animal model of the inherited deficiency in the LDL receptor. Lacking the LDL receptor normally expressed in hepatocytes, these animals rapidly develop atherosclerosis. The feasibility of *ex vivo* retrovirus gene transfer is demonstrated by this model. A partial hepatectomy is performed, removing up to one-third of the liver. The resected portion of the liver is perfused *ex vivo* with enzymes to disperse the hepatocytes, which are then placed in tissue culture and exposed to recombinant retrovirus expressing the LDL receptor. Hepatocytes containing the stably integrated viral DNA are injected through the portal vein back into the liver where they take up residence. This procedure now has been conducted in human patients with the same disorder.

sion of a dysfunctional (mutated) protein. The clinical trial cited above (Crossman *et al.*, 1994) provides the first example of sustained metabolic correction of a genetic defect. The *ex vivo* gene transfer approach likely will be replaced by *in vivo* gene transfer strategies in the future, once problems of vector efficacy, persistence, and immunogenicity are overcome.

Lung. The two most common inherited lung diseases are familial emphysema and cystic fibrosis. Gene therapy strategies have been directed toward the amelioration of both these diseases.

Familial Emphysema. Familial emphysema is a consequence of a defect in the gene encoding the principal endogenous antiprotease, α_1-antitrypsin. This deficiency renders the lungs vulnerable to injury by neutrophil proteases released at sites of inflammation. The α_1-antitrypsin protein is available clinically and is given to patients with the disease. The human gene has been cloned and delivered effectively to the lungs of experimental animals (Canonico *et al.*, 1994). Initial studies in human beings with α_1-antitrypsin deficiency have been approved by the NIH (*see* Table 5–1).

Cystic Fibrosis. Cystic fibrosis is the most common inherited disorder in the Caucasian population, and because most of its morbidity and mortality stems from pulmonary manifestations, it is an ideal model for gene therapy of inherited lung disease. *Ex vivo* gene transfer strategies are not a viable option in the lung. Removal and reimplantation of airway cells is not technically feasible for therapy. Because the target cells in the airway turn over very slowly, retroviral gene transfer, which requires cell division, is very inefficient. In contrast, adenoviral vectors are uniquely suited for this application, as adenovirus has a known tropism for respiratory epithelium. A major potential drawback to the use of adenovirus is the transient nature of gene expression and uncertainty as to whether an adenovirus-induced inflammatory response will allow readministration of the vector. Additionally, airway neutrophils and secretions may decrease transfection efficiency. Nonetheless, a major effort has been launched to develop adenoviral vectors suitable for transducing airway epithelia *in vivo*.

Human studies have been conducted in which adenovirus encoding the cystic fibrosis transport regulator (CFTR) was administered into the nasal epithelium of patients with cystic fibrosis (Zabner *et al.*, 1993). With relatively low doses of virus, normalization of chloride conductance was observed. The major current disadvantage of adenovirus as a vector has been the host response to virally encoded proteins. An inflammatory response to adenovirally transduced cells has been observed in a variety of animal models and in patients, because the vector contains most of the wild-type viral genome. Although the virus has been rendered replication-incompetent by deletion of a subset of viral genes, it still directs the virally transduced cell to synthesize immunogenic viral proteins. Newer versions of the recombinant adenoviral vector may overcome this limitation by attenuating the expression of adenoviral proteins. Engelhardt and colleagues (1994) have shown that alterations of the adenoviral genome in addition to E1 and E3 deletions can decrease the inflammatory response following gene transfer. A temperature-sensitive E2 mutant (ts 125) that preferentially grows at 32° C is introduced into the viral genome so that, when the virus is used to infect cells at 39° C, the mutant E2 protein is less effective in transactivating downstream adenoviral genes that presumably are responsible for inducing the host inflammatory response. In practice, the virus can be propagated in permissive cells (293 cells) at 32° C *in vitro*, and then used to transduce cells *in vivo* at 37° C. Following *in vivo* transduction, the virus is replication-defective (E1 deleted) and less efficient in the synthesis of adenovirus proteins at the elevated body temperature. This results in less inflammation and prolonged transgene expression. Further improvement in the design of adenoviral vectors is under development, including mutations that will remove all or part of the E4 region.

At present, the number of patients treated in all cystic fibrosis gene therapy trials is too small to draw any meaningful conclusions as to efficacy. However, the principles of airway delivery of genetic material are now well established. Future generations of genetic DNA transfer systems, including the adeno-associated virus and liposome systems discussed earlier, likely will offer meaningful benefits not only for cystic fibrosis but also for a variety of lung disorders.

Vasculature. The blood vascular system has been the target of several gene transfer experiments that have demon-

strated the therapeutic potential of gene delivery into this tissue. Both the endothelial cells that line the blood vessels and the smooth muscle cells beneath the endothelium have attracted much attention because of their role in atherosclerosis and the prospect that they might be used to deliver transgene products into the bloodstream. Genetic alterations of these cells might be useful to alter or prevent the process of atherosclerosis, or to deliver vasodilating agents locally or, alternatively, to provide local delivery of anticoagulants.

Ex Vivo Strategies. Initial experiments focused on *ex vivo* gene transfer methods. Wilson *et al.* (1989) demonstrated that canine endothelial cells could be genetically modified *in vitro* by retroviral gene transfer and then transplanted back into the dog as a Dacron® vascular implant seeded with the modified endothelial cells demonstrating transgene expression for over 5 weeks. In another study, cultured endothelial cells from a Yucatan minipig were transduced *in vitro* with replication-defective retrovirus prior to reintroduction into an artery by means of a special double-balloon catheter. By occluding blood flow to a denuded segment of the artery, the catheter provided a temporary protected space where the modified endothelial cells could reattach to the vessel wall (Nabel *et al.*, 1989).

In Vivo Strategies. *In vivo* gene delivery obviates the need for syngeneic cells and will be required for therapeutic applications such as the treatment of atherosclerosis. *In vivo* gene transfer has been achieved using the double-balloon catheter approach with instillation of the DNA delivery system into the protected space of the temporarily occluded vessel. Retroviruses, liposomes, and adenoviral vectors all have been used to target a specific site within a large vessel using this approach.

Atherosclerosis. A variety of genes have been expressed by *in vivo* gene transfer for the purpose of developing useful clinical applications as well as for developing models of pathogenic mechanisms. Vascular cell proliferation and extracellular matrix protein deposition are associated with atherosclerotic narrowing of arteries. Factors that potentially contribute to this process can be studied by overexpressing their genes in arterial segments. For example, when acidic fibroblast growth factor (FGF-1) is ectopically expressed in porcine arteries, the vessel wall becomes thickened (intimal hyperplasia) as a result of smooth muscle cell proliferation (Nabel *et al.*, 1993c). In addition, new blood vessels form within the arterial wall as a result of endothelial cell migration and growth. In contrast, when TGF-β1 is expressed ectopically in the vessel, extracellular matrix synthesis and intimal thickening result (Nabel *et al.*, 1993a). Platelet-derived growth factor B also has been shown to induce intimal hyperplasia following *in vivo* gene transfer (Nabel *et al.*, 1993b). These experimentally induced changes in the vessel wall mimic the changes found in atherosclerotic lesions. Gene transfer thus provides a useful tool to study the effects of agents that may be part of a complex disease process.

Autoimmune Vasculitis. In an attempt to model another arterial disease, autoimmune vasculitis, a foreign histocompatibility gene was delivered to vessel walls by liposome-mediated gene transfer, resulting in a focal immune response at the site of gene transfer that histologically resembles Takayasu arteritis (Nabel *et al.*, 1992). These experiments demonstrate that models of human disease can be developed by introducing specific molecular changes in the blood vessel. These models of arterial disease may be useful in evaluating agents that can block these processes and alter the progression of the disease.

Prevention of Restenosis. In addition to understanding the process by which vascular diseases develop, gene transfer techniques have been developed to treat these diseases. For example, atherosclerotic coronary arteries often can be treated by balloon angioplasty. The narrowed segment of the atherosclerotic vessel is mechanically dilated by the insertion and inflation of a balloon catheter. Although it provides long-term benefits for many patients, this procedure has a high rate of vessel closure (restenosis) within weeks after the dilation. Restenosis occurs, in part, as a result of smooth muscle hyperplasia. Introduction of an adenoviral vector encoding thymidine kinase followed by systemic administration of ganciclovir blocked arterial hyperplasia in an animal model of restenosis (Ohno *et al.*, 1994).

Cancer Gene Therapy

Cancer gene therapies have employed several strategies that rely on unique molecular targets found in cancer cells. Activated oncogenes or mutated tumor suppressor genes are common features of human malignancies. For instance, mutations in the Kirsten-*ras* oncogene, which occur commonly in adenocarcinomas of the lung, are associated with tobacco consumption and may contribute to tumor progression. Mutations in tumor suppressor genes also occur frequently in human cancers. The retinoblastoma p53 gene, which encodes the nuclear protein p53 that regulates cell growth, is the most frequently altered gene in cancer; defects in the function of this suppressor gene and its gene product contribute to unregulated cellular proliferation.

Molecular processes that regulate cell growth, although fundamental to tumor progression, are in general difficult to target with current gene transfer methods for several reasons. Particular oncogenes, such as Kirsten-*ras*, are commonly but not uniformly present in all tumors, even of a given histological type. More important, interruption of a specific oncogene's function or restoration of tumor suppressor gene function would have to be done in every malignant cell, since untreated cells would readily divide. Because most cancers exert their morbidity and mortality through metastatic spread, one is faced with not only targeting every cancer cell but also targeting cancer cells in widespread anatomical locations (bone, liver, lung, brain, *etc.*). Furthermore, many lesions are microscopic metastatic deposits, undetectable by current diagnostic imaging methods. This makes it difficult to assess the efficacy of a new gene transfer method because, in the course of the long follow-up required, it may be unclear whether failure of the treatment resulted from inefficient gene transfer or from any of the many other events that could contribute to ineffectiveness of cancer therapy.

Many tumors acquire a series of genetic defects as they progress. In addition, some tumors arise as a consequence of mutations that result in a gain of function, not loss of function, and thus require ablation of the new activity. For example, chronic myelogenous leukemia occurs as the result of expression of a new chimeric gene product.

Because current gene transfer techniques are unable to achieve a satisfactorily high level of transfer efficiency in an *in vivo* setting, alternative strategies that do not require 100% efficiency of gene transfer have been sought. Two general approaches have evolved that may be effective when only a minority of the tumor cells are transduced: (1) cell-targeted suicide, achieved by directing the synthesis of a toxic metabolite that can permeate the tumor microenvironment, and (2) engineering an immune response to the tumor cells by ectopic cytokine expression or other means for immune recognition or activation.

Cell-targeted Suicide. Conversion of a prodrug to a toxic metabolite by genetically engineering the tumor cell is an attractive way to create an "artificial" difference between normal and neoplastic tissue. This can be achieved by the expression of a gene that confers a dominant, negatively selectable phenotype to the cancer cell, such as cell death imparted by expression of a drug-metabolizing enzyme. A variety of enzymes are capable of performing such a function, and typically kill cells by activation of a relatively nontoxic prodrug to a cytotoxic form (Table 5–2). Greater selectivity in killing malignant cells will be obtained if the transferred gene is not normally found in human beings (*e.g.*, HSV–thymidine kinase), rather than by overexpressing an endogenous gene (*e.g.*, deoxycytidine kinase).

Insertion of the HSV–thymidine kinase (HSV-TK) gene into malignant cells in conjunction with the systemic administration of ganciclovir has become a prototypic gene therapy system that uses the enzyme-prodrug approach. Many investigators have shown that the expression of the HSV-TK gene confers a negative selectable phenotype to cancer cells both *in vitro* and *in vivo*.

Moolten (1986) demonstrated acquired ganciclovir sensitivity in a murine sarcoma cell line transduced with a retroviral vector that produces HSV-TK. The transduced sarcoma tumor cells were 200 to 1000 times more sensitive to ganciclovir than control tumor cells. This finding has been reproduced in several rodent and human cancer model systems including lung cancer, mesothelioma, hepatocellular carcinoma, leukemia, melanoma, and CNS tumor models. The efficacy of this approach varies significantly and may be due to a variety of factors including promoter function, target cells studied, and efficiency of transduction.

The tumoricidal activity of the HSV-TK/ganciclovir system is due to several factors. In dividing cells, the phosphorylated ganciclovir inhibits DNA synthesis. This effect is not confined to cells that are directly transduced with HSV-TK, as neighboring cells are also affected. This phenomenon, which likely occurs as a result of several mechanisms, has been termed the "bystander effect" and has been observed in several tumor types, including CNS tumors (Freeman *et al.*, 1993). Transfer of the phosphorylated ganciclovir between cells ("metabolic cooperation") via gap junctions has been proposed as a possible mechanism. Phagocytosis by neighboring cells of ganciclovir phosphate–containing apoptotic vesicles (from dying transduced cells) also has been proposed. Immune-mediated processes also may account for significant killing of non-transduced cells. In one report, anti-tumor immunity was observed following TK-mediated killing of experimental brain tumors. Whether the tumor immunity is TK dependent, or merely a manifestation of inherent tumor cell immunogenicity, has yet to be established in this rodent model (Barba *et al.*, 1994).

More recently, adenovirus vectors have been used for gene transfer of HSV-TK. Chen *et al.* (1994a) demonstrated regression of experimental gliomas following *in vivo* adenovirus-mediated gene transfer and ganciclovir treatment. The tumor deposits were not completely eliminated by this treatment, however. Tumor cells close to the injection site were more readily transduced than were those distant, as judged by parallel marker gene transfer experiments. Furthermore, these more distant cells escaped ganciclovir toxicity because of a diminished bystander effect attributed to a paucity of gap junctions in the rodent brain tumor cell line employed. This limitation potentially can be overcome in the clinical setting by more precise stereotactic treatment planning (aided by MRI and PET studies) and by multiple tumor injections.

Other approaches have focused on introducing genes that stimulate an immune response to the tumor. Although some have argued that tumor growth occurs as a result of im-

Table 5–2
Enzyme-Prodrug Combinations for Cancer Gene Therapy

GENE	PRODRUG
HSV thymidine kinase (HSV-TK)	Ganciclovir Acyclovir
VSV thymidine kinase	Ara-M
Deoxycytidine kinase	Ara-C Fludarabine 2-Chlorodeoxyadenosine Difluorodeoxycytidine
Cytosine deaminase	5-Fluorocytidine
Nucleoside phosphorylase*	MeP-dR

*Nucleoside phosphorylase is encoded by the *E. coli DeoD* gene, the coding sequence used in this therapeutic strategy.

Key: HSV, herpes simplex virus; VSV, vesicular stomatitis virus; Ara-C, cytosine arabinoside or cytarabine; Ara-M, 6-methoxypurine arabinoside; MeP-dR, 6-methylpurine-2'-deoxyriboside.

mune stimulation, there is little direct evidence to support this hypothesis in most human tumors. Rather, there is a growing body of evidence that suggests that tumor cells express unique determinants that are capable of being recognized by the immune system.

Ectopic Cytokine Expressions. A variety of cytokines have been shown to decrease tumor growth when ectopically expressed on tumor cells or in their microenvironment (Tepper and Mule, 1994). Tumor cells engineered to secrete certain cytokines have been observed to be less able to form tumors when implanted in syngeneic hosts, whereas their *in vitro* growth is unaffected, suggesting that host factors are induced in response to the cytokines that decrease tumorigenicity. Some immunostimulatory agents do not alter the growth rate of the tumor initially, but lead to immunity against tumor growth if the animal is later challenged with wild-type tumor cells. It is apparent that genetically engineered tumor cells elicit a variety of host immune responses depending on the immunomodulatory agent employed. For example, interleukin-4 (IL-4) secretion by a tumor cell elicits a potent local inflammatory response without any effect on distant tumor cells or tumor cells administered at later times. In contrast, granulocyte-macrophage colony stimulating factor (GM-CSF) has little effect on the tumorigenicity, but evokes a potent anti-tumor immunity (Dranoff *et al.*, 1993). In many instances, multiple immune effects are initiated by tumors expressing immunomodulatory agents. This is seen in tumors secreting interleukin-2, where the tumor becomes infiltrated with T lymphocytes, activated macrophages, natural killer cells, neutrophils, and eosinophils. Additionally, a cytokine may have different effects in different tumor types. For example, interleukin-6 can have direct antiproliferative effects, recruit natural killer cells, or serve as an autocrine growth factor, depending on the type of tumor investigated. In many circumstances, it is difficult to distinguish the effects that are induced by the cytokine from the effects mediated secondarily by the other immune effector cells. This has led to a rather empiric approach to cytokine-based cancer gene therapy. The cytokines interleukin-1, -2, -4, -6, -7, and -12, tumor necrosis factor-α (TNF-α), interferon gamma, G-CSF, GM-CSF, and lymphocyte co-stimulatory molecules, all have been shown to induce immune destruction of tumor cells in model systems. Of these, interleukin-2, interleukin-4, TNF-α, interferon gamma, and GM-CSF have been entered into clinical trials using tumor cells genetically engineered to secrete the cytokine (Tepper and Mule, 1994; *see also* Chapter 52).

Immune Enhancement. Other approaches aimed at increasing the immune response to cancer cells have been developed. One such approach is to express highly immunogenic molecules on the surface of cancer cells, such as expression of allotypic MHC antigens. Alternatively, rather than express an exogenous "rejection" antigen, tumor cells may be modified so that the endogenous weakly immunogenic tumor-associated antigens are better recognized. It has been long known that additional "co-stimulatory" pathways distinct from the T-cell receptor are needed to achieve T-cell activation (*see* Chapter 52). The molecules B7-1 and B7-2 stimulate one such pathway. The B7's, whose expression normally is limited to antigen-presenting cells and other specialized immune effector cells, engage specific receptors (CD-28 and CTLA-4) on the T-cell surface in concert with antigen binding to the T-cell receptor. Subsequently, T-cell activation, cell proliferation, and cytokine production ensue, and can lead to the elaboration of antitumor immunity. The absence of a costimulatory signal at the time of T-cell receptor engagement is not a neutral event; rather, it results in the development of tumor-specific anergy, not mere failure to activate the T cell (*see* Chapter 52). Thus, the simple presence of antigens in tumor cells would be expected to produce an immune-tolerant state rather than an immune-responsive state if costimulatory events do not take place. In effect, this is what is seen in most clinical situations where human tumors grow apparently unimpeded by host immune mechanisms. When some tumor cells are provided with co-stimulatory molecules, effective T-cell activation takes place. This has been demonstrated by ectopic expression of B7 on tumor cells, which then are used to stimulate an immune response to the parental tumor cell line.

Several investigators have employed this experimental approach to demonstrate that tumors endowed with B7 co-stimulation ability are able to activate the host immune system to recognize and eradicate tumor cells. Chen *et al.* (1994b) coexpressed B7 and the human papilloma virus E7 rejection antigen in K1735 murine melanoma cells. When injected into syngeneic mice, these cells (E7+B7+) induced a B7-dependent immune response, which resulted in tumor regression. In contrast, E7+B7− tumor cells did not induce an antitumor response. Furthermore, once primed by E7+B7+ cells, mice were capable of rejecting subsequently injected E7+B7− tumor cells. However, these mice were not able to reject the parental tumors, which were B7−. This study also revealed that immune rejection required the presence of CD8+ but not CD4+ T cells.

A similar study by Li *et al.* (1994) suggested the contribution of both CD8+ and CD4+ cells in tumor immunity. A K1735 cell line expressing both MHC class I and II molecules was transfected to express both B7-1 and p97 antigen. The p97 antigen is known to be very immunogenic and to stimulate the production of CD4+ clones specific for this antigen. B7 expression, when coexpressed

with p97, supported the expansion of both the CD8+ cytotoxic T lymphocytes and CD4+ lymphocytes. Furthermore, while CD8+ T cells were the most important effector cells, both cell types were necessary to eliminate established tumor nodules. Clinical experience clearly demonstrates that the mere presence of tumor-associated antigens does not induce an immune response. The implication of these studies is that the ineffectiveness of tumor antigens may be overcome by expressing B7 on the tumor cells. In these and other experiments, the presence of MHC class II molecules on the tumor cell surface, in addition to class I molecules, contributes to the overall immune response, and in particular the CD4+ component of the response. Because most human tumors do not express class II molecules, an effective CD4+ T-cell response may require additional intervention beyond B7 expression. Consequently, providing cytokine stimulation that can provide this effect may be of merit.

The foregoing experiments were performed with what is now known as B7-1. Additional experiments have shown that other molecules (B7-2, and perhaps others) are able to bind the same T-cell receptors as B7-1 (CD-28 and CTLA-4) and activate T-cell co-stimulatory pathways. The differential role of these similar ligands is only beginning to be explored. The temporal course and relative level of their expression are clearly different, as is their ability to be differentially regulated by the same stimuli. A similar level of complexity is emerging for the B7 receptors CTLA-4 and CD-28. Although the differential role of these molecules as they relate to the normal function of the immune system is beginning to be understood, which of the B7's will provide the most effective route to antitumor immunity is unknown (*see* Chapter 52 for a review of cellular mechanisms of immune enhancement and suppression).

T-cell activation, although critically dependent on TCR and co-stimulation pathways, also may be supported by additional functions normally provided by the antigen-presenting cell. Interleukin-12 (IL-12) is secreted by antigen-presenting cells and functions by binding to specific receptors on T cells and natural killer cells. IL-12 induces the production of interferon gamma and enhances the production of a cytotoxic T-lymphocyte response. In one murine tumor model, IL-12, when produced in the microenvironment of a developing tumor nodule, delayed development of detectable tumor nodules (Ohno *et al.*, 1994). IL-12 in this model did not lead to protective antitumor immunity, *i.e.*, tumor development was delayed, but not entirely prevented. Interestingly, the B16-derived BL-6 melanoma cell line is poorly immunogenic yet was able to provoke T-cell activation when supported by this exogenous cytokine. Other investigators have reported that B16 tumor cell lines are not rendered capable of inducing an immune response when transduced to express B7-1. The fact that IL-12 can induce immune responsiveness to a tumor when B7-1 could not suggests that these immunomodulatory molecules may provide different functions. Recently, it has been shown that B7-1 and IL-12 can act in synergy to induce T-cell proliferation and cytokine (interferon gamma and TNF-α) production (see Chapter 52).

Not all of the obstacles to genetically engineered tumor vaccines have been fully identified. Immune tolerance of tumor cells may arise by many mechanisms, including tumor cell secretion of immunosuppressive agents (*e.g.*, TGF-β), and other means to overcome tolerance will need to be devised. Nonetheless, the ectopic expression of genes in cancer cells is a very flexible and powerful tool that likely will improve upon the current therapeutic approach of systemically administered antineoplastic agents (*see* Chapter 51).

Gene Transfer into Hematopoietic Stem Cells

Gene transfer into bone marrow stem cells has been proposed for a variety of inherited and acquired disorders. These include inherited defects in cells produced by the bone marrow (*e.g.*, sickle cell disease, thalassemias, chronic granulomatous disease, and several lymphocyte disorders), as well as acquired illnesses in which marrow-derived cells are secondarily involved (*e.g.*, acquired immunodeficiency syndrome [AIDS] and chemotherapy-induced myelosuppression). The long-term repopulating potential of the bone marrow stem cell also makes it a potentially useful agent for the production and delivery of proteins normally produced by nonhematopoietic cells (*e.g.*, coagulation proteins). The development of bone marrow transplantation has provided substantial precedence for this approach. The growing number of diseases that can be treated effectively by bone marrow transplantation demonstrates the therapeutic efficacy of providing a "corrected" marrow. For example, severe β-thalassemia (an inherited defect in hemoglobin biosynthesis) can be cured by transplantation of bone marrow from a normal donor. The gene therapy equivalent would be to correct the patients' own marrow rather than substitute a "foreign" normal marrow. Because bone marrow can be removed easily and reimplanted, it provides an ideal setting for *ex vivo* gene therapy strategies. The ultimate goal is to be able to transfer genes into hematopoietic stem cells and allow these cells to reconstitute the bone marrow with the selective expression of the transferred gene in a specific hematopoietic cell lineage.

Immunodeficiency Disorders. Gene therapy offers potential treatments for a variety of immunodeficiency disorders. As noted earlier, the first disorder to be treated by gene therapy was a form of severe combined immunodeficiency (SCID) caused by the deficiency of the enzyme adenosine deaminase (ADA). In children with this disorder, the absence of ADA leads to an accumulation of deoxyadenosine triphosphate, which is toxic to lymphocytes; patients develop recurrent life-threatening infections due to defective cell-mediated and humoral immune responses. Current standard therapy includes bone marrow transplantation from an HLA-matched sibling. Although less effective, intravenous replacement of ADA is used in patients who lack a suitable marrow donor. While the first clinical trial of gene therapy for ADA deficiency resulted in clinical improvement, it has not provided a permanent cure. The first patients were treated by repeated gene transfer

into peripheral blood lymphocytes that had been isolated by apheresis. A preferable approach would be to insert the ADA gene into pluripotent hematopoietic stem cells that could reconstitute the immune system with a complete repertoire of immune cells. Such approaches are under development. It has been demonstrated recently that long-term correction of ADA deficiency can be achieved (albeit at low levels) in a rhesus monkey model (Van Beusechem *et al.*, 1992; Bodine *et al.*, 1993).

Leukocyte adhesion deficiency (LAD) is another inherited disorder that results from defective leukocyte function. Patients with this disorder lack cell surface glycoproteins that mediate cell-cell interactions necessary for immune function. Krauss *et al.* (1991) have developed a retrovirus-mediated gene therapy strategy for the treatment of these disorders.

Lysosomal Storage Diseases. Lysosomal storage diseases result from the lysosomal accumulation of cellular material that cannot be degraded, or degraded material that cannot be further processed. Over fifty such disorders are known in human beings and animals. In these disorders, the absence of a particular lysosomal enzyme involved in the breakdown of glycolipids and sphingolipids leads to an increase in lysosome size and number, and secondary derangement of cellular function. The recessively inherited Gaucher disease is typical of the storage diseases in many aspects. Glucosylceramide, a lipid, accumulates in macrophages of affected individuals due to a deficiency of glucocerebrosidase. This results in enlargement of the liver and spleen, destructive bone lesions, and variable central nervous system dysfunction. Several genetic defects are known and there is significant variation in the phenotypic appearance of the disease within a given genotype (*see* Neufeld *et al.*, 1991).

The observation that cultured fibroblasts from an affected individual could be "cross-corrected" by coculture with normal cells that secrete the enzyme led to the development of replacement therapy. Although intravenous administration of the deficient enzyme is not highly effective in patients, replacement therapy has demonstrated that enzyme-deficient cells are able to take up exogenously produced enzyme. Alternatively, transplantation of an affected patient with normal bone marrow cells can offer clinical improvement in some cases of lysosomal storage disease. The transplanted hematopoietic cells are able to deliver normal enzyme to affected tissues. Cells capable of making the normal enzyme can transfer the secreted enzyme to a recipient cell by a receptor-mediated endocytosis pathway or via direct contact-mediated transfer. This capacity for cell-to-cell transfer of lysosomal enzymes via receptor-mediated endocytosis has been demonstrated in a number of animal models, including a murine model of β-glucuronidase deficiency (Bosi-Charlos *et al.*, 1993) and a feline model of α-mannosidosis (Walkley *et al.*, 1994). Although bone marrow transplant may be therapeutically useful in some circumstances, its utility is diminished by the availability of suitable marrow donors and the immunosuppressive risks associated with transplanting allogeneic bone marrow. Gene transfer methods that may overcome these shortcomings are being developed. By engineering the patient's marrow to express the desired enzyme, the patient's own leukocytes could deliver normal enzyme. In one proposed treatment strategy, bone marrow would be harvested from the patient and the "corrected" gene inserted in *in vitro* culture. Reinfusion of the manipulated marrow cells would lead to the long-term replacement of the enzyme without the need for immunosuppressive agents. Several investigators have effected retrovirus-mediated gene transfer into marrow cells from animals and human beings and demonstrated that long-term production of the desired enzyme is achievable.

Drug Resistance Genes in the Treatment of Cancer. The mechanisms by which cancer cells are able to survive the cytotoxic effects of chemotherapy are well described for a number of chemotherapeutic agents. These mechanisms include the expression of genes that are able to inactivate or eliminate the toxic drug (*see* Chapter 51). Although these genes currently serve to limit the effectiveness of many chemotherapy regimes, it is possible that they might be redeployed to have the opposite effect, that is, to protect normal tissues from the toxic effects of chemotherapy. One gene in particular has received much attention in this regard, the multidrug resistance (MDR-1) gene encoding the multidrug transporter protein (also known as P-glycoprotein). This transmembrane protein is capable of pumping a wide variety of chemotherapeutic agents (*e.g.*, adriamycin, vinca alkaloids, epipodophyllotoxins, and taxol) and other drugs out of cells, thus protecting them from the agents' toxic effects (Gottesman *et al.*, 1994). Many cancers display a dose-dependent sensitivity to chemotherapy, whereby larger doses of chemotherapy lead to greater tumor regression and improved survival (*see* Chapter 51). This is best illustrated by testicular cancers, which are highly curable when treated aggressively. Unfortunately, toxicity to normal tissues, especially the bone marrow, limits the use of larger doses of chemotherapy in many cancers. To overcome this, autologous bone marrow transplantation has been employed to rescue the bone marrow from the toxic effects of high-dose chemotherapy. In some cancers (*e.g.*, breast cancer and testicular cancer), relapse after standard therapy can be treated by harvesting uninvolved normal bone marrow prior to high-dose chemotherapy. The stored autologous marrow is then reinfused to rescue the patient from therapy-induced marrow ablation. Such high-dose chemotherapy with autologous bone marrow transplantation is now standard therapy for relapsed testicular cancer. Capitalizing on this concept, a gene therapy-based strategy has been proposed whereby

the MDR-1 gene would be used to render the bone marrow resistant to the toxic effects of the chemotherapy (Gottesman *et al.*, 1994).

Although gene transfer into marrow stem cells leads to transgene expression in only a few percent of hematopoietic cells, successive cycles of chemotherapy can be used to enrich for transduced marrow cells. This approach may be applied to cancers that demonstrate a steep dose-response to chemotherapy and where myelosuppression is the dose-limiting toxicity.

Gene Therapy for Infectious Diseases.

The failure of conventional antibiotics to treat many types of serious pathogenic agents effectively, most notably the human immunodeficiency virus, and the availability of unique molecular targets in these pathogens have encouraged the exploration of gene therapies for infectious diseases.

AIDS. Nabel *et al.* (1994) and Malim *et al.* (1992) have used a dominant negative mutant protein in designing a gene transfer strategy for the treatment of AIDS. The rev protein, produced by the human immunodeficiency virus, is a regulatory protein necessary for viral replication. It binds to a specific viral RNA motif (rev response element, RRE) and promotes the synthesis of new viral proteins. Studies in experimental models have shown that by introducing a mutant *rev* gene, the HIV-infected cell produces an altered rev protein. This protein, called Rev M10, is capable of binding the same motif as the normal rev, but is not functional in promoting the synthesis of new viral proteins. Consequently, Rev M10 competitively inhibits the activity of the normal rev protein and ultimately attenuates HIV replication.

Immunization. By an entirely different approach, gene transfer can be employed to drive the synthesis of an antibody with predetermined specificity. This would eliminate the need to rely on a variable or unpredictable immune response to a vaccine (particularly in immune-compromised patients) and could be used to direct the synthesis of the antibody to a specific site. Chen *et al.* (1994b) recently have described a single-chain antibody with specificity for the gp120 HIV protein that can be delivered by gene transfer. They have shown that human CD4+ T lymphocytes can be transduced to express this antibody intracellularly, and that cytopathic syncytium formation and HIV-1 production were inhibited, although not eliminated.

PROSPECTUS

Human gene therapy, although still in the infant stages of development, offers the possibility for major advances in the prevention and treatment of myriad diseases. Gene therapy brings an entirely new paradigm for the treatment of disorders stemming from missing or defective genes, whether they are inherited or acquired. Furthermore, this technology likely also will evolve for the treatment of "nongenetic" illnesses, where the tissue-specific synthesis of a protein can be used for therapeutic benefit. The identification of new genes related to specific diseases will broaden the scope of applications. Currently, however, the clinical application of gene therapy is more limited by the availability of suitable gene transfer methodology than by the identification of suitable targets for genetic alteration. However, as increasing numbers of investigators address these issues, better reagents likely will emerge. Furthermore, a better understanding of the pathophysiological processes will permit the design of physiologically appropriate interventions. It is to be hoped that increased collaboration among physicians, molecular biologists, and cell biologists will result in the development of highly integrated approaches to this new form of therapy.

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